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# New Advances in 3D Models to Improve Diabetic Keratopathy Research: A Narrative Review

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**Abstract**

Diabetic keratopathy (DK) is a common ocular complication of diabetes mellitus (DM), affecting almost half of all diabetic patients. It is characterized by delayed healing of epithelial wounds, reduced corneal sensitivity, and persistent epithelial defects, which, in turn, significantly impair vision and quality of life. The limited understanding of its pathogenesis and the lack of effective treatments highlight the urgent need for more physiologically relevant experimental models. The three-dimensional (3D) models currently available provide valuable information on the pathophysiology of DK, although none of them yet fully reproduce the diabetic corneal phenotype complex. After a brief overview of corneal anatomy, the present review aims to systematically analyze the current 3D in vitro models developed for the study of DK, in terms of tissue architecture, presence of diabetic stimuli, and ability to replicate pathological traits.

**Keywords:** cornea; 3D corneal models; diabetic keratopathy; diabetes mellitus



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## 1. Introduction

Diabetes mellitus (DM), a chronic systemic disease, is one of the most common and deadly pathologies in the world, increasing globally. It is estimated that 537 million (10.5%) persons worldwide are currently managing the disease [1]. DM is caused by an innate or acquired insulin deficiency, leading to alterations in glucose metabolism and a persistent condition of hyperglycemia. Chronic hyperglycemia is associated with the development of several chronic eye diseases [2], including diabetic keratopathy (DK), which occurs in 47–64% of DM patients [3]. DK is a degenerative disease of the cornea whose pathogenic mechanisms are not yet fully understood. It is clinically manifested by persistent corneal epithelial defects, superficial punctate keratopathy, alteration of the normal wound-healing mechanism, and reduced corneal sensitivity, sustained by aberrant oxidative stress (Figure 1) [4,5]. These issues can impair patients' vision and overall quality of life [2].

Despite extensive scientific efforts, effective treatments for DK are still limited, mainly offering symptomatic relief rather than tackling the root cellular and molecular issues. Therefore, developing three-dimensional (3D) models that closely mimic the cornea's structure offers significant potential for unraveling the intricate biology of diabetic corneal complications and could be a crucial tool for testing future therapies. The 3D models

provide a more accurate representation of the corneal microenvironment than traditional in vitro methods, leading to a deeper understanding of cellular behavior and treatment efficacy. Therefore, a thorough examination of existing 3D models for the diabetic cornea is crucial for comprehending the complex biological features underlying this diabetic complication. This review aims to survey current 3D models of DK. We will explore the latest advancements in diabetic keratopathy modeling while also addressing the challenges in fully replicating the disease's complexity. Recognizing the pressing need for precise models, this discussion emphasizes the substantial influence that these advanced 3D models could have on understanding and managing this prevalent diabetic eye condition.

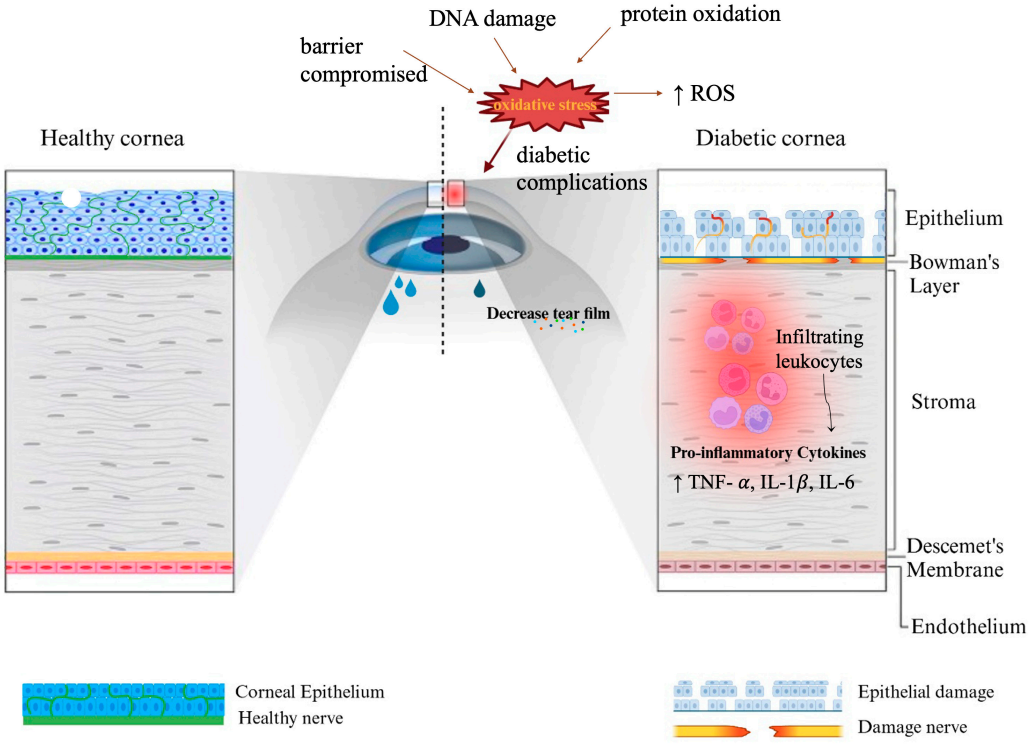



**Figure 1.** Structural and inflammatory alterations of the diabetic cornea compared to the healthy cornea: The healthy cornea shows an orderly and correctly stratified structure, with regularly distributed epithelial cells. The corneal nerves (in green) appear intact, suggesting normal innervation and corneal sensitivity. Eye hydration is normal, guaranteed by adequate tear film. The diabetic cornea is subject to oxidative stress, contributing to the reduction in the tear film, leading to dry eye. The epithelium shows signs of cellular damage, compromised corneal nerves (yellow–red), and stromal inflammation. This figure was created with BioRender (https://BioRender.com).

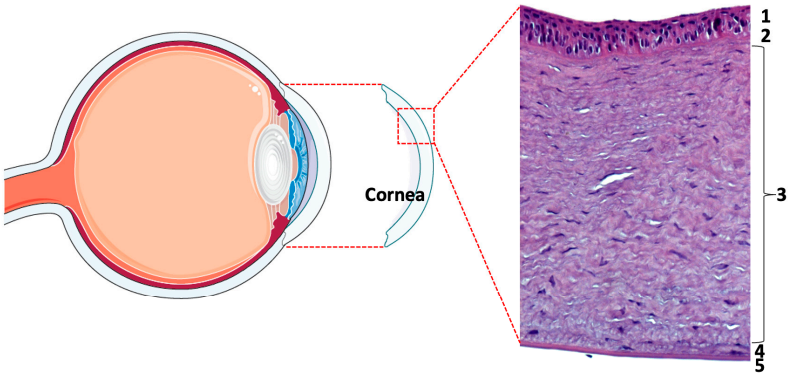
## 2. Anatomy of the Cornea and Structural Organization

The cornea is a transparent, avascular, and highly organized structure located in the front of the eyeball. It plays a key role in focusing light on the retina. In addition to its optical function, the cornea is the main physical and biological barrier against external agents, contributing to the protection of the intraocular environment from trauma and infections [6,7].

Anatomically, the human cornea consists of six main layers, arranged from outside to inside: epithelium, Bowman's membrane, stroma, the pre-Descemet's layer/Dua's layer, Descemet's membrane, and endothelium [8]. Each of these layers performs specific functions that help maintain corneal transparency, structural integrity, and homeostasis (Figure 2).

The epithelium is the outermost layer of the cornea, about 50–60 µm thick in adult humans. It is a multicellular layer, composed of four to six layers of unkeratinized stratified

squamous epithelial cells, attached to an epithelial basal membrane composed of laminins, collagen IV, and other proteoglycans [9,10]. The epithelial cells are morphologically distinct in basal columnar cells, wing cells, and surface squamous cells. At the base of the epithelial layer lie basal epithelial cells, of cuboidal shape, forming a monolayer anchored to the underlying basal lamina by means of hemidesmosomes, and being mitotically active, guaranteeing the continuous renewal of the epithelium. The wing cells, so called because of their wing-like cross-sectional shape, are derived from basal cell differentiation and are organized in two or three layers. These cells have narrow lateral and intercellular junctions that contribute to the structural integrity of the epithelium. Surface squamous epithelial cells stratified in two or three layers, and not keratinized, act as a permeability barrier against pathogens, toxins, and foreign molecules. These cells are polygonal in shape and have extensive apical and micropile microvilli. These cells are also joined by close-junction complexes that limit the access of tears to intercellular spaces [11].




| CORNEAL LAYERS | FUNCTIONS |
|---|---|
| (1) Epithelium | • Physical barrier from external damage<br>• Optical function, contributing to refractive power of the eye<br>• Immunological functions through the presence of Langerhans cells |
| (2) Bowman's Layer | • Mainteins the corneal shape, offering protection for the stroma |
| (3) Stroma | • Mechanical strength to cornea<br>• Transparency of cornea<br>• Main refracting lens |
| (4) Descemet's Membrane: | • Resting layer for endothelial cells |
| (5) Endothelium | • Maintains corneal hydration and clarity by pumping out excess water. |

**Figure 2.** Morphology and functions of the healthy cornea. Schematic and histological image show the main layers of the cornea.

The corneal epithelium is maintained by the continuous renewal activity of limbal epithelial stem cells (LESCs). LESCs are adult stem cells characterized by high proliferative potential and robust self-renewal capabilities. Anatomically, these stem cells are housed in the limbus, a transition zone between the cornea and the conjunctiva, within a specialized microenvironment known as the niche. This niche, which includes structures like limbal epithelial crypts and Vogt palisades, is a highly controlled location within the limbus that provides the necessary physical and biochemical signals to regulate LESC function [12].

Located below the epithelium, Bowman's membrane is an acellular layer composed of randomly arranged collagen fibrils [13,14], which help the cornea to maintain its shape [15]. It has a thickness of about 8–12 µm in humans, and it tends to become thinner with age.

The thickest layer of the cornea is the stroma, which accounts for more than 80%. Its transparency results from the careful organization of collagen fibers (mainly type I) and extracellular matrix (ECM) [16]. These collagen fiber lamellae are laid out in regular layers interspersed with keratocytes, widely distributed resident stromal cells involved in the maintenance, repair, and regeneration of stromal tissue [17]. In response to damaging stimuli, keratocytes can transform into a (myo)fibroblast phenotype and release growth factors and cytokines [18]. They also play an important role in the maintenance of stromal homeostasis by synthesizing collagen, glycosaminoglycans, and matrix metalloproteinases (MMPs). Following injury or infection, superficial and mid-stromal keratocytes may undergo apoptosis (depending on the severity of the lesion), while distal keratocytes from the wound can transform into mobile and mitotic active fibroblasts. Under the

persistent influence of factors such as TGF-β 1/β 2, derived from tears and epithelium, these fibroblasts can further differentiate into alpha myofibroblasts of smooth muscle, which produce high levels of disorganized extracellular matrix and subsequently lead to the development of scar stromal opacities, often responsible for vision loss [19–21]. The stroma is also home to a dense nervous network. The corneal nerves, derived from the trigeminal nerve, branch into plexuses that cross the anterior stroma to the inside of the subepithelial nervous plexus and the epithelium [22].

In 2007, the first evidence of a distinctive layer within the deep stroma, located before the Descemet membrane, was provided, called the "pre-Descemet stromal layer", also known as the Dua-Fine layer or pre-posterior limiting plate. This thin layer shows a high mechanical strength, despite having an average thickness of about 10 µm (varying between 6 and 16 µm), and is composed mainly of collagen types I and VI, rich in elastin, to a greater extent than the other corneal layers. It is also virtually cell-free and airtight. In its peripheral portion, fenestrate and branched structures are observed that constitute the core of the trabecular network, suggesting a possible role in the regulation of intraocular pressure and in the pathophysiology of glaucoma. Its presence has also been documented in some animal species [23].

The Descemet membrane is an acellular basal plate, dense and relatively transparent, and rich in collagen IV, collagen VIII, laminins, and proteoglycans such as perlecan and nidogen [24]. It separates the posterior stroma from the endothelium and participates in corneal homeostasis by regulating its hydration.

The innermost layer of the cornea is formed by a single layer of strongly cohesive polygonal cells, with a thickness of about 4 µm [25]. The upper surface of the cells adheres to the Descemet membrane through hemidesmosomes and tight junctions located on the apical side of the endothelial cells. In this way, the corneal endothelium acts as a barrier preventing the permeability of solutes and the escape of liquids. It also acts as a functional pump regulating the transport of water, glucose, and other solutes from the aqueous humor to the stroma, thanks to the presence of aquaporins (AQPs) and the Na/K ATPase pump, both of which regulate the passage of fluids through the endothelium [26]. The presence of tight junctions and ion pumps in endothelial cells facilitates fluid exchange through the posterior surface of the cornea [27], thus playing a key role in regulating corneal hydration and maintaining tissue transparency. A balanced ion flow between the endothelium and aqueous humor prevents the appearance of stromal edema, which would impair the optic function of the cornea. In adults, the average corneal endothelial cell density is ~3000 cells/mm$^2$, with a percentage of hexagonal cells of 75%. However, cell loss due to aging, disease, injury, or surgery may compromise transparency, as HCECs have limited proliferative capacity [28]. In response to minor damage, surviving cells may enlarge and migrate to cover the injured area, thus maintaining endothelial structure and function [29].

## 3. Diabetic Keratopathy (DK)

Diabetes-related eye diseases, which tend to appear early in the course of the disease, are a major cause of blindness in both developed and developing countries [30]. Diabetes mellitus (DM) has been shown to negatively affect the ocular surface through complex neurobiological and neuroinflammatory interactions [3]. In addition, ocular surgeries such as corneal transplantation, vitrectomy, and cataract surgery are additional risk factors for the development of corneal epithelial lesions in diabetic patients. In the past, the focus was mainly on diabetic retinopathy and cataracts. However, today, with the evolution of widely available and in-depth diagnostic techniques, diabetic keratopathy (DK) has attracted increasing interest from the medical and scientific community [31,32]. Despite the

numerous studies conducted on corneal morphological changes in patients with diabetes, the pathogenic mechanisms underlying DK are not yet fully understood [33,34].

In the last 30 years, the prevalence of diabetes has increased exponentially. It is estimated that between 46% and 64% of diabetic patients develop DK [5]. In light of the rapid increase in the prevalence of diabetes mellitus and the frequent occurrence of DK in patients with long-term disease [35–37], it is foreseeable that this condition may be more prevalent than other eye diseases. Although the clinical manifestations of DK may resemble those of neurotrophic (NK) keratopathy [38], the two pathologies differ in their pathogenic mechanisms: NK is determined by purely neuropathic damage, while DK is mainly supported by inflammatory and immune-mediated processes, which are not yet fully clarified [39,40].

DK, or diabetic corneal epitheliopathy [41,42], is a chronic condition affecting the cornea and has clinical features including recurrent epithelial erosions, delayed epithelial regeneration, neurotrophic corneal ulcers, corneal hypoesthesia, corneal edema, decreased corneal sensitivity, and superficial punctate keratopathy (SPK). The latter is characterized by sparse areas of punctate corneal epithelial loss, causing photophobia, foreign body sensation, tearing, redness, irritation, and reduced visual acuity [43]. These pathogenic changes do not respond to available conventional treatments, and they affect quality of life [2,36,44–48], leading to impaired visual acuity or permanent loss of vision.

Corneal complications, reported in Table 1, range from mild to severe and include epithelial defects, corneal thickness, corneal cell density, biomechanics, corneal erosions, lacrimal secretion, and corneal sensitivity [46].

**Table 1.** Corneal complications associated with diabetes mellitus.

| Complication | Description and Main Mechanisms | References |
| --- | --- | --- |
| Increased corneal thickness (CCT) | Observed in diabetic patients, especially those with long-standing disease. Caused by stromal edema, epithelial and endothelial dysfunction, AGE formation, and chronic hyperglycemia. Associated with high HbA1c, hyperglycemia, and retinal complications. | [2,4,48,49] |
| Reduced corneal sensitivity | Due to degeneration of corneal nerve fibers. Impairs epithelial integrity and healing. Associated with prolonged and recurrent lesions. | [4,10,50–55] |
| Corneal erosions | Caused by epithelial barrier dysfunction and reduced by regeneration. Increased risk of infection. May cause stromal disorganization and corneal thinning. | [4,56–59] |
| Epithelial defects and impaired wound healing | High susceptibility to erosions and ulcerations, with delayed healing. Can lead to scarring, visual acuity loss, and neovascularization. | [32,33,39,40,59–66] |
| Reduced cell density | Disruption in balance of proliferation, differentiation, and death. Increased risk of infections and erosions. Basal epithelial cell density reduced, likely due to innervation loss. Endothelial density remains comparable to that of healthy subjects. | [44,67,68] |
| Altered tear secretion | Lacrimal gland dysfunction reduces secretion. Reduction in the lipid layer and mucin layer, and impairment of the aqueous component of tears. Neuropathy, microangiopathy, and hyperglycemia involved. Goblet cell loss reduces the mucin layer. | [69–72] |
| Biomechanical alterations | Structural changes in the stroma affect elasticity and mechanical strength. Structural change can alter visual quality and refractive properties. | [73] |

- Corneal thickness

Among the ocular alterations induced by diabetes mellitus, one of the first observable manifestations at the eye level is thought to be the increase in corneal thickness [4,49]. Some studies indicate that this thickening is more pronounced in subjects with a duration of

the disease longer than ten years [50]. Several clinical studies have reported an increase in central corneal thickness (CCT) in diabetic patients compared to healthy subjects. As a result of epithelial and endothelial dysfunction of the diabetic cornea, stromal edema largely contributes to the increase in thickness [2]. The formation of advanced glycation end-products (AGEs) and the resulting collagen crosslinking also contribute to thickening. In addition, clinical studies on vascular components of the disease have shown that elevated HbA1c, hyperglycemia, and severe retinal complications were associated with increased CCT in patients with diabetes [4,49,50].

- Corneal sensitivity

In diabetes mellitus (DM), the degeneration of corneal nerve fibers is associated with a reduction in corneal sensitivity [51], a condition that can evolve into keratopathy [52]. This is because corneal nerves play a crucial role in protecting the eye, helping to maintain epithelium integrity, and facilitating wound-healing processes [53,54]. Some studies have observed that diabetic patients with corneal complications often present prolonged and difficult-to-heal epithelial lesions [4], probably due to an altered adhesion between the epithelium and the basal membrane [55]. Nerve alterations and basal membrane abnormalities may contribute to the development of recurrent epithelial defects [10]. For this reason, corneal sensitivity assessment may be useful in the early diagnosis of diabetic peripheral neuropathy and/or keratopathy [56].

- Corneal erosions

Corneal erosion is one of the main clinical manifestations in patients with diabetic keratopathy. In patients with diabetes mellitus (DM), the healing of these lesions is particularly difficult due to impaired epithelial barrier function and reduced regenerative capacity of the epithelium, exposing the cornea to an increased risk of infection and, consequently, causing persistent corneal erosion and edema [4]. In addition, it has been observed that corneal erosion can contribute to thinning of the cornea, due to the disorganization of the corneal stroma, which disturbs the fibrillar dispositions of collagen and the consequent alteration of the extracellular matrix [57–60].

- Epithelial defects and wound healing

The corneal epithelium is essential for maintaining the transparency and physiological balance of the cornea, as well as acting as a barrier against infection and trauma. In diabetes mellitus (DM), however, an increased susceptibility to spontaneous corneal lesions such as erosions and epithelial ulcerations is observed [34,41,61]. Corneal lesions in diabetic patients tend to heal more slowly, being persistent and resistant to traditional therapies [33,62]. Therefore, an effective and rapid re-epithelialization is fundamental to avoid microbial infection that may compromise corneal transparency and visual function. Persistent corneal epithelial defects from diabetic keratopathy often lead to severe visual defects [40]: stromal scars, reduced visual acuity or permanent loss of vision, and corneal neovascularization [63–65]. In patients with diabetes mellitus, corneal re-epithelialization is more complex, due to morphological alterations of the epithelium, such as disorganization of cellular layers, reduction in endothelial cells, sectoral thinning, variations in cell shape and size, and the presence of bubbles and surface debris [60]. As seen from several recent articles, the healing of corneal wounds is a process regulated by complex mechanisms, which include epithelial migration and proliferation, the interaction between epithelial and stromal cells, and the activation of specific growth factors and cytokines: TGF-β, EGF, HGF, OGF, IGF, NGF, KGF, PDGF, timosina-β4, IL-6 e 10 [40,66,67].

- Corneal cell density

The maintenance of corneal epithelial density depends on a balance among proliferation, migration, differentiation, and cell death. In diabetic patients, this balance is often compromised, increasing susceptibility to infection and erosion [68]. Some studies have shown a reduction in basal cell density in subjects with diabetes, suggesting that this deficit may be related to the loss of corneal innervation [45]. However, as far as endothelial cells are concerned, patients with type 2 diabetes show values similar to those of healthy subjects [69].

- Tear secretion

In diabetes mellitus (DM) a close association with lacrimal gland dysfunction has been observed, contributing to the development of dry eye syndrome (DES). Diabetic patients show typical symptoms such as burning, foreign body sensation, and temporary fluctuations in visual acuity. In addition, an altered composition of the tear film can cause reduction in the lipid layer. Corneal neuropathies induced by hyperglycemia and microvascular damage compromise the feedback mechanisms that regulate lacrimal secretion, favoring neurotrophic lesions. Clinical studies have shown that, in subjects with DM, lacrimal secretion is reduced compared to healthy controls [70], while the decrease in conjunctival caliciform cells is responsible for the reduction in the mucin layer [71]. The reduced corneal nerve density compromises the secretion of the aqueous component of tears. Chronic hyperglycemia (leading to insulin deficiency) adversely affects the proliferation of acinous cells of the lacrimal gland and corneal epithelium [72]. Diabetic microangiopathy can also interfere with lacrimal secretion, damaging the vascularization of the lacrimal gland [73].

- Biomechanics and crosslinking

Some research has suggested that the stroma provides most of the biomechanical strength of the cornea, although all layers of the cornea show some biomechanical properties. The cornea is an elastic structure with high amounts of collagen, which possesses biomechanical characteristics fundamental for the refraction of light. Any structural change may compromise its mechanical integrity and visual quality [74]. The diabetic phenotype may alter the biomechanical properties of the cornea, thus also affecting its optical function.

DK is a complex condition involving multiple components of the ocular surface, including the epithelium, corneal nerves, tear film, endothelium, and partially the conjunctiva. The hyperglycemic environment also causes significant changes in the stroma, with increased stiffness and thickening due to collagen crosslinking mediated by AGEs [57,59,75]. In this way, they not only hinder healing but can also compromise the accuracy of the intraocular pressure measurement. Endothelial dysfunction is also one of the clinical features of DM, with morphological alterations and reduced cell density contributing to increased corneal thickness [76]. At the molecular level, chronic hyperglycemia activates a number of harmful metabolic pathways, including stimulation of the polyol pathway, increase in reactive oxygen species (ROS), activation of protein kinase C (PKC), and accumulation of advanced glycation end-products (AGEs) [3]. The latter form as a result of an excess of glucose, impairing the cellular migration towards the site of damage, slowing the process of repair and healing of corneal lesions.

Oxidative stress plays a central role in the pathogenesis of DK, together with the reduction in nerve growth factor (NGF), which impairs corneal sensitivity and reparative capacity [77]. Metabolic dysregulation and activation of the NF-κ B inflammatory pathway, mediated by pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, further contribute to tissue damage [78]. In particular, IL-1β and TNF-α induce MMP-9, aggravating the degradation of the epithelial basement membrane [79,80]. In case of hyperglycemia and oxidative stress, signaling pathways such as ERK [81], PI3K/Akt [42], and TGF-β [82]

are activated and disrupt the repair of corneal epithelial cells; they are also involved in epithelial dysfunction, fibrosis, and the reorganization of the extracellular matrix. These are mechanisms that contribute to the onset and progression of diabetic keratopathy.

Although current therapeutic strategies focus mainly on stimulating healing through the use of growth factors and artificial tears, the targeted treatment of pathogenic mechanisms remains a challenge. Neuroprotective agents, such as the use of NGF, have shown promise in promoting corneal nerve fiber regeneration and epithelial repair [83], underlining the importance of a deeper understanding of the molecular mechanisms behind DK for the development of more effective therapeutic approaches. Moreover, a deeper understanding of the molecular mechanisms and morphological alterations behind DK is essential for the development of more effective therapeutic approaches, and the 3D models represent promising tools for better understanding the characteristics of the disease.

## 4. Overview of 3D Corneal Models

In recent years, 3D models have shown great potential to improve our understanding of the healing, regeneration, and pathogenesis processes of corneal diseases. In particular, 3D in vitro models clarify cell and extracellular matrix (ECM) interactions by accurately reproducing complex environments in vivo [84], providing cells with physiological access to nutrients and oxygen [85]. Numerous attempts have been made to develop in vitro 3D cornea models. To make a complete corneal 3D model, all major relevant cell types should be included: epithelial, stromal, endothelial, and nerve cells, derived from primary cells or stem cells, integrated into an extracellular matrix (ECM). The presence of ECM in a 3D culture is known to affect cells' proliferation, differentiation, and survival [86–88]. The main 3D technologies applied to the study of the cornea include organotypic models and organoids.

### *4.1. Organotypic Models*

Organotypic models recreate the typical cell architecture and stratification of the cornea, facilitating the study of interactions among epithelial, stromal, endothelial, and neuronal cells. Several combinations of organotypic cultures have been developed over the years (Table 2). An example is a 3D stacked construct of stromal fibroblasts and human corneal epithelial cells (hCECs) cultured successively on the surface to mimic the 3D environment [89] and capable of producing ECM on their own. Moreover, in this model, the ability of corneal fibroblasts to produce and organize their extracellular matrix in the presence of ascorbic acid [89] is investigated. After all, it is well known that one of the endogenous properties of ascorbic acid is to promote an increase in collagen secretion and the thickness of the assembled ECM [90]. Sharif et al. (2018) [91] developed a 3D model, the first of its kind to mimic interactions between stromal and neuronal cells, to be used in the future as a tool to investigate corneal defects. In this model, stromal cells self-produce their extracellular matrix, thus allowing both cell–matrix and cell–cell interactions between fibroblasts and nerve cells to be studied. In this regard, it is clear how this model only reproduces stroma–nervous interactions, leaving out essential components such as epithelium and endothelium, thus limiting the representativeness of the complete corneal architecture. The authors proposed this model to study pathologies such as DK, keratoconus, and Fuchs' dystrophy. In another model, human induced neural stem cells (hiNSCs) were seeded on silk sponges placed at the periphery of the insert, while a stacked construct of corneal, stromal, and epithelial cells was placed in the center. In particular, hiNSCs can grow under co-culture conditions with multiple cell types and show a faster and more robust neuronal differentiation than induced pluripotent stem cells [92]. Interestingly, the thin silk films used as scaffolds to support stroma growth and epithelial layers are

biodegradable material, which degrades slowly in vitro and does not contract as compared to collagen [93,94].

**Table 2.** Comparison of 3D corneal organotypic models.

| Model | Scaffold Material | Cell Type | Duration | Measured Parameters | References |
|---|---|---|---|---|---|
| Stacked fibroblasts and hCECs | None (self-produced ECM) | Human corneal epithelial cells (hCECs), stromal fibroblasts | Not specified | ECM production | [86,87] |
| Stromal–neuronal co-culture | None (self-produced ECM) | Corneal fibroblasts, neuronal cells | Not specified | Cell–matrix and cell–cell (stromal and neuronal cells) interactions; model for DK, keratoconus, and Fuchs' dystrophy | [88] |
| hiNSC peripheral silk sponge with central stacked construct | Silk sponge | hiNSC, stromal, and epithelial corneal cells | Not specified | Neuronal differentiation, co-culture compatibility | [89–91] |
| ALI co-culture with DRG neurons | Silk films | Human corneal stromal stem cells (hCSSCs), hCECs, DRG neurons | 28 days | Morphological stability, innervation, transparency, epithelial maturation | [92,94] |

Silk film biomaterials have been designed to meet functional requirements, including transparency, mechanical integrity, biocompatibility, and slow biodegradation, to meet potential in vivo utility [93]. In the present work [93], 3D stacked and porous silk films were successfully prepared, which supported cell viability and ensured a mechanically robust and simplified architecture for the development of corneal devices [95]. The results show that silk films offer a favorable environment for cell alignment and native ECM synthesis. The use of silk-based biomaterials, beyond sutures, is an area that has only recently been explored, but with promising clinical prospects. This represents the first successful demonstration of a multilayer film construct assembled with more than two layers of cultured cells.

Furthermore, controlling the rate of degradation is important in tissue design, so that it coincides with tissue growth. For example, silk fibroin fibers retain more than 50% of their mechanical properties after two months of in vivo implantation [96].

Given the importance of innervation, Siran Wang et al. [97] have made a three-dimensional model of the human cornea using a silk scaffold that integrates human corneal stromal stem cells, human corneal epithelial cells, and dorsal root ganglion (DRG) cells in co-culture at an air–liquid interface (ALI). The system was maintained for 28 days, and a robust scaffolding of silk proteins ensured morphological stability and transparency for the entire life of the crop. This study allowed for improvements in the study of innervation, corneal tissue development, corneal diseases, and drug screening.

Among the few co-culture studies involving corneal cells and neurons [98], layers of collagen hydrogel were used to mimic the lamellar organization of the cornea. However, these models failed to accurately reproduce the alignment of stromal cells or the multilayer stratification typical of corneal epithelium. Silk films, designed with surface patterns and functionalized with DRG, are effective in facilitating alignment, proliferation, and matrix production by human corneal stromal cells [99]. In addition, silk can also be used as a sponge to support neuronal growth and the formation of neural connections. So, researchers used thin films of superimposed silk protein as scaffolds to support the epithelial (hCEC) and stromal (hCSSC) layers of the cornea, while a surrounding porous silk sponge seeded with DRG neurons facilitated neuronal growth. The results confirm that corneal sensory

nerves are crucial for maintaining the viability, metabolism, and cellular supply of corneal tissues [100]. It has also been observed that the innervation is located on the upper surface of the scaffold, contributing to the transparency and proper functioning of the stroma. Each layer of silk film used improves the function and vitality of cultivated tissues. In addition, the air–liquid interface environment has proven to be crucial in promoting the maturation of the corneal epithelium and supporting the development of an extended and well-organized neuronal innervation under in vitro conditions. Therefore, both the presence of innervation and exposure to the ALI are important for the achievement of cellular and tissue maturity of the corneal epithelium.

### 4.2. Organoid Technology

Organoid technology, an advanced aspect of 3D cultures, has been rapidly developing since 2009 [101]. The term "organoids" refers to structures similar to miniature organs [102], cultivated in a laboratory environment and requiring a specific 3D microenvironment capable of supporting their development and cell differentiation. Under in vitro conditions, 3D organoid cultures have the ability to self-renew and self-organize, reproducing the main structural and functional characteristics (such as absorption, secretion, and excretion [102,103]) and genetics of native organs [102]. These structures may be derived from pluripotent stem cells (PSCs), including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [104]. A significant advantage of organoids is their ability to maintain cellular characteristics stably over time [105], even during cultivation and after numerous passages in culture without alterations at the genomic level [103], guaranteeing reliable and reproducible results. In addition, organoids are generable from patients' cells, making it theoretically possible to develop highly personalized therapies [106]. Stem cell-derived organoids represent a promising alternative for the regeneration of damaged tissues, reducing donor demand for organ transplants. They also provide the possibility of in vitro testing of the compatibility of tissues to be transplanted, increasing the chances of success and reducing the risk of rejection [107,108]. Recently, Chuwei Lu et al. [109] offered an overview of organoid technology, focusing on the development of corneal limbal organoids (CL-ORGs). The first human CL-ORG was developed in 2017 [110] from human induced pluripotent stem cells (hiPSCs) derived from human fetal fibroblasts. These organoids contained the three main types of corneal cells. Human CL-ORGs were later generated from human ESCs and LESCs [111], increasing the potential applications of these CL-ORGs in mimicking corneal development, disease modeling, drug screening, regenerative medicine, and treatment of limbal stem cell deficit (LSCD). In fact, in an animal model of rabbits affected by LSCD, it has been shown that transplanted in vitro-cultured CL-ORGs can effectively restore the healthy ocular surface [111]. Kazunari Higa et al. [112] have also produced organoids with the function of limbal epithelial progenitor cells from the central cornea, where in vivo there are no stem cells. It has been hypothesized that, during organoid culture, the central corneal epithelial cells may undergo a dedifferentiation process, acquiring a phenotype similar to that of limbal epithelium, supporting the regenerative capacity of transplanted organoids in a rabbit limbal deficit model. CL-ORGs, also known as "mini-corneas", are three-dimensional in vitro corneal-like models originating from PSCs or limbal epithelial stem cells (LESCs) cultured in a controlled 3D environment. These organoids show anatomical and genomic characteristics similar to the human cornea [113] and contain major cell types, identified by expression of specific markers of epithelium, stroma, and endothelium [110]. During the COVID-19 pandemic, CL-ORGs were used as an in vitro model to study the interaction between SARS-CoV-2 and ocular surface cells, proving useful in assessing eye-level implications [114,115]. Despite their potential, essential physiological components such as innervation, vascularization,

and immune regulation are currently lacking. In addition, the cultivation process requires longer times [116] and strict growing conditions, the alteration of which may compromise the quality and efficiency of the model [117].

Xichen Wan et al. [118] established a long-term functional 3D human epithelial organoid derived from ASCs (adult stem cells), capable of replicating the characteristics of human corneal epithelium and serving as an ex vivo model for the study of functional regulation and modeling of dry eye disease (DED). Meanwhile, Susaimanickam et al. [113] reported an efficient and simpler method to generate complex three-dimensional corneal organoids obtainable from both human ESCs and iPSCs. During the process of differentiation, stem cells form self-organized structures similar to mini-corneas, which include retinal primords, corneal cells, an outer covering similar to a primitive eyelid, and accessory tissues similar to the ciliary margin area, maturing progressively over 15 weeks. The mini-corneas reproduced the early stages of eye development in vitro, showing characteristics comparable to those of the adult cornea.

Most differentiation protocols using ocular cells and organoids derived from hiPSCs focus on a single tissue-specific lineage, which fails to reflect the tissue complexity of eye development. For this reason, Isla-Magrané et al. [119] realized a model of multiocular organoids. They used a two-step method, differentiating hiPSC cultures within three weeks in multizone ocular progenitor cells (mzOPCs). Then, mzOPCs developed into different 3D multiocular organoids composed of multiple cell lineages, including corneal cells. Multiocular organoids showed a variable composition, with complex retinal pigmented epithelium (RPE)–retina–cornea models or simpler combinations containing retina–RPE, retina–cornea, or RPE–cornea. For corneal organoids, the presence of p63+ cells together with PAX6 suggests the development of a primitive epithelial surface [120], similar to those obtained from human limbal cells [111]. Organoids with corneal or conjunctival characteristics have emerged in mzOPC cultures, distinguishable by specific markers such as CK3, AQP1, and N-Cad for the cornea [121] and CK5, CK19, and p63 for the limbo-conjunctive [122]. Note that corneal organoids with a conjunctival predominance contained caliciform cells expressing mucin 1. In addition, corneal organoids also contain mesenchymal cells [110,113] derived from neuronal crest cells responsible for the production of stromal collagen types I and V and the formation of a Bowman layer-like structure. Table 3 reports the main characteristics of the described organoids.

**Table 3.** Comparison of corneal organoid models.

| Model | Cell Types | Culture Duration | Measured Parameters | References |
|---|---|---|---|---|
| CL-ORG from hiPSCs | Corneal epithelial, stromal, and endothelial cells | Not specified | Disease modeling, drug screening, and gene editing and tissue replacement. | [105] |
| CL-ORG from ESCs and LESCs | ESCs and limbal epithelial stem cells, central corneal epithelial cells | Not specified | Mimic corneal development, disease modeling, drug screening, limbal stem cell deficiency treatment, and regenerative capacity | [104,106,107] |
| CL-ORG used in SARS-CoV-2 studies | PSC-derived epithelial/stromal cells | Not specified | Virus interaction with ocular surface cells | [109,110] |
| Human epithelial organoid | ASC-derived corneal epithelial cells | Not specified | Dry eye disease modeling | [113] |
| CL-ORG from ESCs and iPSCs | ESC- and iPSC-derived cells (corneal, retinal, accessory) | 15 weeks | Early stages of eye development, adult-like corneal features | [108] |
| Multiocular organoids | Retina, RPE, cornea, conjunctiva | 3 weeks to differentiation + maturation | Marker expression: CK3, AQP1, and N-Cad for the cornea; CK5, CK19, and p63 for the limbo-conjunctive | [114–117] |

## 5. Exploring 3D Models for DK

Significant progress is being made in developing 3D models that more closely mimic the morphofunctional characteristics of the human cornea [123–126]. An overview of the different strategies employed by research groups for 3D modeling of DK is outlined in Table 4. This compilation provides a useful benchmark for comparison and could guide future optimization of the models. Priyadarsini et al. [127], developed a 3D model capable of reproducing the corneal stromal environment of DM patients. Briefly, they isolated human primary corneal fibroblasts from healthy donors and patients with type 1 (T1DM) and type 2 (T2DM) diabetes. These cells were grown for 4 weeks on transwell inserts and stimulated with vitamin C to promote secretion and self-assembly of the ECM. The stroma model showed structural and biochemical characteristics similar to those of the cornea in vivo, making it suitable for the study of corneal alterations induced by diabetes. In particular, T1DM and T2DM constructs showed a significant increase in thickness and cell density as compared to the healthy group. Moreover, T1DM and T2DM stroma models reported upregulation of fibrotic markers such as $\alpha$-smooth muscle actin, alterations in key metabolites of the glycolysis and tricarboxylic acid cycle pathways, and significant mitochondrial damage as compared to the healthy stroma model. Very recently, Maugeri et al. [128] realized a 3D organotypic model of diabetic epitheliopathy by culturing human corneal epithelial cells (hCECs) derived from donor corneas in ALI culture. The hCECs were cultured under normal glucose (NG) levels (6.2 mM glucose) or in the presence of 25 mM high glucose (HG). The corneal epithelial barrier cultured under HG levels showed well-established molecular and cellular characteristics of DK, including epithelial defects and inflammation, with increased expression of IL-1$\beta$, TNF-$\alpha$, p-NF-kB, COX-2, MMP-2, and MMP-9. Cui et al. [129] realized a novel full-thickness biomimetic corneal model. They seeded collagen membranes containing corneal stromal cells (CSCs) on the upper well and immortalized corneal endothelial cells (CEnCs) on the undersurface. Then, dorsal root ganglion organoids (DRGOs) were cultured on a collagen scaffold, and after seven days, corneal epithelial cells were seeded onto the upper surface of the transwell insert and cultured in ALI. The model, exposed to 65 mM glucose to induce the pathological characteristics of DK, showed nerve bundle breakdown, weakened barrier function, heightened inflammation, and oxidative damage. Moreover, transcriptomics studies revealed that high glucose levels suppressed genes involved in axon and synapse formation, while enhancing pathways related to immune response and oxidative stress. Deardorff et al. [92] realized a three-dimensional construct that replicates the characteristics of diabetic corneal neuropathy observed in vivo, thus studying the morphological and functional effects of hyperglycemia on corneal innervation. The model integrates human corneal epithelial cells (hCECs), stromal cells (hCSSCs), and sensory neural stem cells derived from human induced neuronal stem cells (hiNSCs), grown on silk scaffolds to enable cell alignment and adhesion [97]. This system, characterized by a long-term co-culture (42 days), allowed sensory neurons to innervate the upper epithelial tissue. To simulate the diabetic condition, the model was exposed to high concentrations of glucose (35 mM and 45 mM), causing a significant reduction in nerve fiber density and length—which is a clinical feature associated with diabetic neuropathy—and a decrease in cell density in epithelial and stromal cells. In addition, an increase in the expression of interleukin-1$\beta$ (IL-1$\beta$), indicative of a pro-inflammatory environment induced by hyperglycemia (45 mM), was observed. The corneal epithelium is known to be one of the main sources of IL-1$\beta$ in the cornea, in response to wounds or infections [130]. Several studies have highlighted a role of IL-1$\beta$ and the IL-1Ra receptor, suggesting that this molecular pathway may play a key role in wound-healing processes in the diabetic cornea [131].

In diabetes, chronic hyperglycemia is closely linked to oxidative stress, which contributes to impairment of corneal epithelium function and regeneration. These effects were investigated in an in vitro reconstructed 3D normal human corneal epithelial (3D-HCE) tissue model exposed to ultraviolet radiation (UV), hydrogen peroxide, the vesicating agent nitrogen mustard, or desiccating conditions to reproduce dry eye disease conditions [132]. The data obtained indicated the validity of the 3D-HCE reconstructed corneal tissue model in reproducing the main functions of the corneal epithelium, particularly in response to oxidative stress conditions. An increased expression of pro-inflammatory cytokines and genes associated with stress response, alterations in the epithelial barrier, and reduced wound-healing capacity were observed.

There have been few studies dealing with interactions between the corneal stroma and nerves, which are fundamental in the pathogenesis of diabetic corneal neuropathy. Several studies agree that corneal nerve dysfunction is commonly seen in DM. Therefore, Priyadarsini et al. [133] cultured human corneal fibroblasts from healthy donors and patients with type 1 and type 2 diabetes for 3 weeks on 3D scaffolds. Subsequently, the SH-SY5Y human neuroblastoma cells differentiated into human neural cells, were seeded over the 3D construct, and then were co-cultured with fibroblasts to simulate corneal innervation. Numerous studies have already confirmed the effectiveness of using SH-SY5Y neuroblastoma cells and their ability to differentiate into functionally mature neurons [134]. In this study, the priority was to obtain viable neurons with morphology similar to that of mature human neurons. The results showed that fibroblasts isolated from diabetic patients showed altered production of ECM, with changes in collagen fiber composition and organization compared to healthy fibroblasts. In particular, a marked overexpression of collagen III was found in the control of T2DM under almost all conditions, while levels of α-SMA did not reach significance in any group. An increase in the expression of Tubulin β III was also observed, as well as a significant overregulation of the expression of IGF1 and IGF1-R in the control of T2DM. Finally, the relevant modulation of both Tubulin β III and Nestin in co-culture conditions indicates an active interaction between stromal and neuronal cells, underlining significant differences in crosstalk between a healthy stroma versus a T1DM/T2DM stroma. Interactions among stroma, epithelium, and nerves are critical to maintaining a healthy cornea.

Overall, these 3D in vitro models are revolutionizing preclinical research by better mimicking the physiological complexity of human corneal tissues compared to traditional 2D cell cultures. They offer a promising platform for disease modeling, drug discovery, and personalized medicine. However, significant challenges must be overcome to fully translate the findings from these advanced models into meaningful clinical applications, such as identifying novel biomarkers or creating effective therapeutic screening platforms.

**Table 4.** Main experimental approaches used for the 3D reproduction of DK in vitro models.

| Corneal Layer | Cell Type | Technology | DK Model | Molecular Markers | Nerve Function | Thickness | Cell Density | Advantages | Limitations | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Stroma | Primary fibroblasts of the human cornea from healthy donors and patients with T1DM and T2DM | Transwell inserts | T1DM and T2DM | ↑ α-SMA, mitochondrial damage, altered glycolysis/TCA | Not specified | ↑ | ↑ | Structural and biochemical characteristics similar to those of the cornea in vivo | No innervation | [127] |

**Table 4.** *Cont.*

| Corneal Layer | Cell Type | Technology | DK Model | Molecular Markers | Nerve Function | Thickness | Cell Density | Advantages | Limitations | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Epithelium | Human corneal epithelial cells (hCECs) derived from donor cornea | *ALI* | 25 mM glucose | ↑ IL-1β, TNF-α, p-NF-kB, COX-2, MMP-2, MMP-9 | Not specified | ↓ | ↓ | Reproduces epithelial inflammation in DK | No stroma or nerves | [128] |
| Stroma and endothelium | Corneal stromal cells (CSCs), corneal endothelial cells (CEnCs), and dorsal root ganglion organoids (DRGOs) | Scaffold of collagen. *ALI* | 65 mM glucose | ↓Axon/synapse genes, ↑ immune/oxidative stress pathways | ↓ | ↓ | ↓ | Full-thickness biomimetic corneal model, innervation | No epithelium interaction | [129] |
| Corneal innervation, stroma and epithelium | Human cornea epithelial cells (hCECs), stromal cells (hCSSCs), and sensory neural stem cells derived from induced human neuronal stem cells (hiNSCs) | Scaffold of silk | 35–45 mM glucose | ↓ Nerve fiber density/length, ↑ IL-1β | ↓ | ↓ | ↓ | Functional co-culture with sensory neurons | No epithelium interaction | [92] |
| Corneal innervation, stroma | Human corneal fibroblasts from healthy donors and patients with type 1 and 2 diabetes; SH-SY5Y human neuroblastoma cells | 3D scaffold | T1DM and T2DM | ↑ Collagen III, IGF1, IGF1-R, Tubulin βIII, Nestin | ↓ | ↓ | ↓ | Neurons similar to mature human neurons | No epithelium or endothelium interaction | [133] |

↑ Increase ↓ Decrease.

## 6. Conclusions

The development of 3D in vitro models for DK research represents a significant paradigm shift in our approach to understanding this complex ocular complication. These models offer valid opportunities ranging from multicellular architecture reproduction to mechanistic insights into disease progression useful for drug screening and efficacy testing. Furthermore, corneal 3D models are a powerful tool for advancing research and drug development in ophthalmology while adhering to the 3Rs principle (Replacement, Reduction, and Refinement), which aims to minimize animal use in science. These models offer a replacement for animal testing, providing a human-relevant system for assessing drug toxicity and efficacy. They also enable the reduction in animal numbers needed for later-stage studies and help filter out ineffective or harmful substances earlier. Therefore, corneal 3D models represent an ethical and scientifically superior alternative that is helping to transition research from animal-based to human-relevant systems. Despite their advantages, 3D in vitro models face several limitations. The cornea shows a complex in vivo environment, which includes dynamic factors such as tear film composition, blinking mechanics, temperature fluctuations, and systemic metabolic influences that are difficult to replicate in vitro. The absence of vascular perfusion and immune cell recruitment may limit the models' ability to fully capture inflammatory responses and healing processes observed clinically. Moreover, standardization challenges across different laboratories and model systems complicate data interpretation and reproducibility. Therefore, standardization efforts across the research community will be crucial for establishing validated protocols and enabling meaningful cross-study comparisons. The development of reference standards and quality control metrics will enhance the reliability and reproducibility of these models.

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## Abbreviations

| | |
|---|---|
| 3D | Three-dimensional |
| 3D-HCE | Three-dimensional normal human corneal epithelial |
| AGEs | Advanced glycation end-products |
| ALI | Air–liquid interface |
| AQP | Aquaporin |
| CCT | Central corneal thickness |
| CEnCs | Corneal endothelial cells |
| CL-ORG | Corneal limbal organoid |
| CSCs | Corneal stromal cells |
| DED | Dry eye disease |
| DES | Dry eye syndrome |
| DK | Diabetic keratopathy |
| DM | Diabetes mellitus |
| DRG | Dorsal root ganglion |
| DRGO | Dorsal root ganglion organoid |
| ECM | Extracellular matrix |
| ESCs | Embryonic stem cells |
| hCEC | Human corneal epithelial cell |
| hCSSC | Human corneal stromal cell |
| HG | High glucose |
| hiNSC | Human induced neural stem cell |
| hiPSC | Human induced pluripotent stem cell |
| IL-1β | Interleukin-1β |
| iPSC | Induced pluripotent stem cell |
| LESC | Limbal epithelial stem cell |
| LSCD | Limbal stem cell deficit |
| MMP | Matrix metalloproteinase |
| mzOPC | Multizone ocular progenitor cell |
| NG | Normal glucose |
| OS | External segment |
| NGF | Nerve growth factor |
| NK | Neurotrophic keratopathy |
| PKC | Protein kinase C |
| PSC | Pluripotent stem cell |
| ROS | Reactive oxygen species |
| RPE | Retinal pigmented epithelium |
| SPK | Superficial punctate keratopathy |
| T1DM | Diabetes mellitus type 1 |

| | |
|---|---|
| T2DM | Diabetes mellitus type 2 |
| UV | Ultraviolet radiation |

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