 doi:10.20944/preprints202009.0756.v1

Review

Development of *in vitro* corneal models: opportunity for pharmacological testing and legislative aspects

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Abstract: Human eye is a specialized organ with complex anatomy and physiology, because it is characterized by different cell types with specific physiological functions. Given the complexity of the eye, ocular tissues are finely organized and orchestrated. In the last few years many *in vitro* models have been developed, in order to meet the 3Rs principle (Replacement, Reduction and Refinement) for eye toxicity testing which is necessary to ensure that the risks associated with ophthalmic products meet appropriate safety criteria and are clearly labelled. *In vitro* preclinical testing is now a well-established practice of significant importance for evaluating the efficacy and safety of cosmetic, pharmaceutical, and nutraceutical products. Along with *in vitro* testing, also computational procedures, herein described, for evaluating the pharmacological profile of potential ocular drug candidates including their toxicity, are in rapid expansion. In this review the ocular cell types and functionality are described providing an overview about the scientific challenge for the development of three-dimensional *in vitro* models.

Keywords: 3D *in vitro* models; eye research; *in silico* analysis; eye anatomy

1. Introduction

Human eye is a deeply specialized organ with a singular anatomy and physiology, containing several structures with specific physiological functions. Given the complexity of the eye, ocular tissues are finely organized and orchestrated. As a result, optimal visual function is maintained while passage of solutes, fluids and also drugs is highly controlled.[1]

1.1. Structure of the human eye

Among adults, the size of the human eye differs by a few millimeters. In particular, it might be considered a fibrovascular sphere with a circumference of about 70-85 mm and an anteroposterior diameter of 22-27 mm.[2] The eye is characterized by three layers, which enclose many anatomical structures. The outermost layer is the fibrous tunic; it is composed by the cornea and sclera and provides both shape and support to the eye. The middle layer (uvea or vascular tunic), includes the iris, pigmented epithelium, choroid and ciliar body.[3] The innermost layer is represented by the retina, which is a neurosensory structure fundamental for the vision process. Briefly, the vision process is initiated by conduction of the light through clear cornea; then, the light reaches the pupil circumscribed by the iris, that is a sort of shutter or camera diaphragm.[4] Finally, the light crosses the crystalline lens and the adjacent vitreous gel, ultimately impacting the retina, where the optic nerve transmits the visual impulse to the brain.[2] Cornea, lens and vitreous are transparent tissues, that allow the passage of light to the retina with minimal distortion or absorption. However, this physiological conduction of light occurs exclusively in the healthy eye, while an impaired vision process under pathological conditions (and also in the elderly) has been reported.[5]

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1.2. The ocular chambers

The human eye consists of three different ocular chambers: the anterior chamber, the posterior chamber and the vitreous chamber.[2] Aqueous humor fills both anterior and posterior chambers; this liquid, produced by the ciliar body in the posterior chamber, is transported by the trabecular meshwork of the Schlemm's canal into the anterior chamber. Importantly, any change in the amount of aqueous humor can modify the intraocular pressure (which normally is 10-20 mmHg[6]), thus increasing the risk of development of many pathological conditions, including glaucoma.[7]

1.2.1. The anterior chamber

Anatomically, the anterior chamber is made of iris and cornea. The iris is a colored circular muscle with a central aperture named "pupil". In the human eye, iris is characterized by a variable pigmentation; in particular, melanocytes deeply contribute to the eye color producing both eumelanin (dark melanin) and pheomelanin (light melanin).[7]

Cornea is a transparent, refracting and nonvascularized structure, characterized by collagenous fibrils dispersed in a mucopolysaccharide layer and balanced hydration (also referred to as "deturgescence"). Cornea and opaque sclera, its non-transparent extension, are inelastic structures that provide mechanical support to the eye globe.[8] In particular, sclera is more permeable than cornea (about 10 times more), is poorly vascularized and characterized by the presence of both mucopolysaccharides and collagen. The regular and rigid structure of both cornea and sclera protects the eye from the external environment. Moreover, cornea is coated by the tear film, which is produced by both mucous membrane conjunctiva and lacrimal glands.[2] Its composition ensures cornea hydration, acts as lubricant, provides nutrients and limits the entering of toxins or particles into the eye.[9] However, many pathological conditions, environmental factors and/or toxins may affect the integrity of the cornea, leading to its degeneration, vascularization and opacification.[1]

Cornea plays also a fundamental role in ocular drug administration, as consists of many static (i.e., stroma, epithelium, Bowman's layer and endothelium) and dynamic (i.e., lymph circulation and choroidal blood) barriers.[10] Among the static barriers, epithelium (which is characterized by efflux pumps expressed on the cell membrane) represent the main layer that limits the transport of drugs through the cornea.[11] Epithelium is a stratified (about 50 μM in thickness), non-keratinized and squamous tissue. It is composed of 2-3 layers of flattened cells and a single layer of columnar basal cells separated by intercellular spaces (≅ 15 nm).[10] Moreover, human epithelium contains tight-junctions that reduce the absorption of macromolecules and hydrophilic compounds; in addition, the stroma restricts access to numerous lipophilic molecules, as it is characterized by high content of hydrated collagen.[12] Finally, both the tear film and lymphatic clearance reduce ocular drug absorption.[10]

Together with epithelium, also the blood aqueous barrier (BAB) contributes to the control of solutes and fluids into inner ocular tissues.[13] Both endothelial cells of the blood vessels within the iris and the non-pigmented cells of the ciliary epithelium take part of the BAB. As the corneal epithelium, also this cell layer contains tight-junction complexes which limit the non-specific traverse of solutes and drugs through the anterior chamber of the eye. In particular, it has been demonstrated that many macromolecules (> 40 kDa) cannot pass through iris blood vessels, while their passage is allowed through fenestrated capillaries of the ciliary body.[1] In contrast, small lipophilic molecules can be rapidly eliminated from the anterior chamber through uveal blood circulation, while the aqueous humor turnover deeply contributes to elimination of small hydrophilic molecules in the anterior chamber.[2] For instance, the clearance rate of pilocarpine (≅ 66 kDa) is 13.0 μL/min while that of the small hydrophilic molecule inulin (3-5 kDa) is close to the aqueous humor turnover value, which is estimated to be 1-2% of the anterior chamber volume per minute.[14]

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1.2.2 The posterior chamber

This eye chamber is located between the zonular fibers and iris; in the center there is the crystalline lens which represents the second light refracting structure together with the cornea. Crystalline lens is anchored to the ciliary body by means of the zonular fibers, which are composed by hundreds of fibers that regulate accommodation (focusing) through continuous modification of shape of the lens.[12] Crystalline lens is transparent due to the absence of light-scattering organelles in fiber cells.[5] As well-described, many disorders may affect the transparency of lens. Among them, cataracts, ageing, environmental factors and also drugs are the most common causes of lens opacification.[15]

1.2.3. The vitreous chamber

Vitreous chamber (also known as the "vitreous body") is delimited by crystalline lens (anteriorly) and retina (posteriorly). This eye chamber represents the largest tissue in the human eye, and contains few cells and a jelly-like, sticky and aqueous gel.[5] Retina is the neurosensory structure of the eye, which is involved in photoreception.[2] Indeed, this layer is characterized by the presence of cone and rod photoreceptors, that transduce light signals into electrical signals. Then, electrical signals are integrated by various interneurons, that are amacrine, horizontal and bipolar cells. Finally, the processed signals reach the optic nerve that transmits signals to the brain.[16] According to its crucial role in regulating the vision process, many pathological conditions affecting retina may progressively lead to disturbed vision or blindness. In particular, both genetic mutations and toxins may irreversibly contribute to retinal cell damage.[12, 17]

Inside the retina, there are small blood vessels that form a very complex vascularization network. Indeed, nearby to the photoreceptor layer, there is the retinal pigment epithelium (RPE), which is a melanin-pigmented layer that ensure retinal integrity and form the outer blood-retina barrier (oBRB).[18] The inner BRB (iBRB), instead, is composed by the non-fenestrated retinal capillary endothelial (RCE) cells. Both oBRB and iBRB guarantee a continuous exchange of nutrients[1] but show low permeability to proteins and hydrophilic compounds,[19] thus representing an additional barrier for ocular drugs. In particular, RPE allows selective exchange of nutrients between choroid and retina. Indeed, in their apical site, RPE cells highly express Na^+/K^+-ATPase pump that regulates intracellular homeostasis of Na^+ and K^+ ions.[20] In addition, RCE covers the lumen of retinal capillaries and protects the retina from circulating compounds, thus limiting the passage of large molecules trough this barrier.[21] Finally, the posterior side of the human eye is characterized by an additional vascularized layer, the choroid, which provide nutrients to the retina [2]. The Bruch's membrane is the innermost layer of choroid and contains the RPE cells.[21]

1.3. The lacrimal system

The lacrimal system is responsible for both the production and drainage of the tear film covering the cornea, which prevents ocular dehydration and protects the eye from environmental stressors.[22] The main lacrimal gland, located in the lacrimal fossa, represents the crucial component of this apparatus; in addition, the accessory lacrimal glands of Wolfring and Krause also contribute to the production of the tear film.[23] The lacrimal system's drainage, instead, is composed by both superior and inferior lacrimal canaliculi that carry the tear film into the lacrimal sac, up to the nasolacrimal duct to the inferior nasal meatus.[2]

The tear film contains a well-balanced electrolyte composition, and a complex mixture of mucin, lipids, proteins (such as secretory immunoglobulins, lipocalin, lactoferrin and peroxidase) and growth factors.[24]

The TFOS DEWS II Tear Film Subcommittee recommended a two-phase model of the tear film, which has a lipid layer overlying a muco-aqueous phase. Wax and cholesteryl esters (non-polar

 doi:10.20944/preprints202009.0756.v1

lipids) make up the majority of the tear lipid layer and these are spread onto the muco-aqueous layer by an underlying layer of polar lipids, including (O-acyl)-u-hydroxy fatty acids and possibly phospholipids.[25] The superficial lipid layer (0.1 μM in thickness) is crucial for stabilization of the film, reducing also evaporation of the underlying layer; the second layer is composed by two level: the middle layer (up to 10 μM in thickness), also known as the aqueous layer, that consists of proteins and water soluble salts; the internal layer (0.2-1 μM in thickness), which is mainly produced by the conjunctival cells, and characterized by high levels of high-molecular weight glycoproteins (especially mucin). This second layer adheres to the corneal epithelial cells, contributing to both retention and distribution of the central aqueous tear film on the cornea.[1]

2. *In vitro* ocular models

2.1. Opportunity and legislative aspects

Given the complexity of eye anatomy reported above, a key issue in the regulation of ophthalmic products is to identify their relative risks to eye tissue.[24] For this reason, recognizing and classifying the potential risk of commercial products is highly necessary. Eye toxicity testing is therefore necessary to ensure that the risks associated with products meet appropriate safety criteria and are clearly labelled. *In vitro* preclinical testing is now a well-established practice of significant importance for evaluating the efficacy and safety of cosmetic, pharmaceutical, and nutraceutical products.[26] The realization and development of increasingly sophisticated experimental models, in particular those based on reproducible models in three-dimensional cell cultures, reduce the costs of experimental procedures, obtaining predictive information on the ocular tolerability and efficacy of a product, severely limiting the *in vivo* experimentation on animals.[27] The development of novel *in vitro* approaches is firstly linked to the campaigns carried out in this decade by few associations, which strongly ask for a reduction in animal testing.[28] This has prompted regulatory bodies to find alternative methods and solutions to animal testing in the various sectors (cosmetic, pharmaceutical and nutraceutical). The new approach to toxicology was the engine that gave birth to several promoters of these new investigation methodologies. Thus, the principle of the 3Rs (Refine, Reduce, Replace) has been considered a stimulating opportunity to improve *in vitro* methods, even if nowadays it is not possible to completely abolish animal experimentation.[29] Scientific world gave the introduction of experimental in vitro models a strong impulse, implementing EU validated alternative methods in compliance with Good Laboratory Practice (GLP). The new Medical Devices Regulation (MDR 745/2017), is a further opportunity for companies operating in the preclinical sector.[30] Furthermore, the exponential evolution of *in vitro* technologies and in particular the potential of three-dimensional systems (human tissues 3D reconstructed *in vitro*), often proved to be more relevant and predictive than monolayer cell models. These three-dimensional models, at first, were quickly developed under the regulatory push, in order to replace animal models. They have been included in numerous OECD (Organization for Economic Co-operation and Development) validation studies. In a short time, they became increasingly predictive of the response and were rapidly adopted in preclinical research. The advantages of using experimental *in vitro* models with 3D cell cultures is that they have an organization and structure very similar to tissue *in vivo*.[31] At the basis of their realization there are sophisticated technologies and they are produced in Good Manufacturing Practice (GMP), and therefore standardized. They show reproducible results in functionality and in biological responses to reference substances. They can be assimilated to "simplified models" but with a very specific purpose in which it is possible to define the direct mechanism on the target organ. Moreover, they quantitatively and reproducibly evaluate numerous experimental parameters that contribute to create a series of demonstrative tests. This aspect has great importance both for the regulatory classification and for the intellectual protection of a product as well as for scientific communication.

2.2. Conventional 2D models

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The investigation of basic developmental or differentiation processes can be studied using primary or immortalized human cells deriving from the cornea, retina, and conjunctiva to understand and clarify pathophysiological conditions or to set up models in order to reproduce specific disease models and to perform toxicological and pharmacological studies. Epithelial cells, keratocytes, fibroblasts, and trabecular meshwork cells are critical components required for the normal function of the ocular cell system. Atypical cell proliferation and regulation within the ocular cell system contributes to the development of disorders such as corneal inflammation, proliferative retinopathy, macular degeneration, glaucoma, and retinoblastoma. Cell culture techniques allow to evaluate the physiology of the different ocular cell types outside the living organism in reproduced conditions that mimic, as closely as possible, the environment of the tissue or organ which they derive. Among the possible applications, we can mention: a) the investigation of the physiological processes of cell life and of the response to exogenous treatments in controlled environment;[32] b) the evaluation of the effect of various chemical molecules and drugs on specific cell types; c) the study aimed to the generation of reconstructed tissues (e.g., artificial corneal tissues). In the living organism, cells are kept vital thanks to the supply of nutrients, supported by the vascular system which, through the capillary vessels, nourishes the tissue and abolishes those harmful molecules deriving from cell metabolism. *In vitro* the role of the vascular system is substituted by the culture medium, a highly nutritious liquid medium. It consists of basic substances, such as glucose, amino acids, vitamins, minerals, fundamental for the physiological processes of cells, and animal serum, which supports cell growth and proliferation. Thanks to this culture conditions, ocular cell-culture models offer several advantages over animal experimental models, including a higher reproducibility, easier handling, and reduced costs, but still giving the possibility to study mechanistic processes of physiological or pathological altered pathways. Corneal cells can be directly exposed to test samples (chemicals or environmental matrix samples) at low and relatively defined concentrations. In this regard, although distribution and excretion phenomena (which occur in *in vivo* exposure) do not occur, the bioavailable concentration of the test sample must be taken into account even in the *in vitro* models. The interaction of the sample with the cells allows a very rapid evaluation (even by hours) of the effect on cell activities and also allows to verify the reversibility of the response. Animal cell cultures can be used as a low-cost, rapid screening tool for toxicological and pharmacological evaluation of chemicals. Moreover, the problems deriving from inter-species variability are avoided if cells of human origin are used.[33] However, primary cells usually can only be used for limited passages before starting to lose their normal physiology and structural characteristics. Immortalized cell lines remain functional for a several passages but have increased the likelihood of developing chromosomal abnormalities, reduced expression of key markers, or abnormal growth.[32] However, there are also some limitation in the use of cell cultures. The *in vivo-in vitro* translation causes the loss of specific cell-cell interactions, histological characteristics of the tissue of origin and the components involved in homeostatic regulation (especially those of the nervous and endocrine systems). There are also metabolic alterations with drop in some enzymatic levels (e.g. cytochrome P450) or changes in metabolic cycles, so that the energy metabolism of cells is largely based on glycolysis. Due to the strong selection in favor of the most actively proliferating cells, the culture also suffers a loss of differentiated properties.

2.3. Advanced corneal 3D models

There are a wide variety of *in vitro* methods that have been developed, to date. Organotypic and cell-based testing methods are not compatible with real human eyes. Differences between species caused by the use of animals' eyes may lead to excessive and insufficient prediction of eye irritation. The monolayer cell cultures employed in cell testing do not realistically reproduce the complicated 3D environment of real tissue. Artificially rigid and flat surfaces of culture articles may alter cell metabolism and intrinsic functionality. To overcome these errors, 3D models equivalent to the human cornea have been developed. Recently, 3D models of human cornea have been developed, based on normal human cells which are grown on an inert polycarbonate insert (Figure 1).

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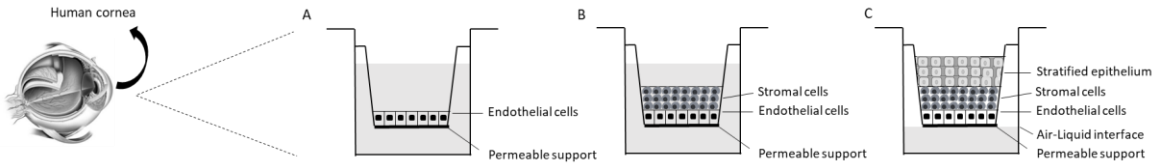

Figure 1. T Schematic representation of a 3D in vitro corneal model: (A) Corneal endothelial cells grow on a permeable support up to confluence. (B) A 3D matrix containing stromal cells grows on top of the endothelial layer. (C) Epithelial cells are seeded on the stromal layer; then, exposure to air-liquid interface results in a stratified epithelium.

These tissues, are validated and standardized and each batch is derived from a single donor, giving a huge advantage in terms of accuracy and reproducibility of the data obtained. The human cornea is formed by epithelium, stroma and endothelium. Although ideal 3D models equivalent to human cornea should have all the three components of the cornea, only the human cornea-like epithelium (RhCE) has been presently developed, due to technical limitations. However, the corneal epithelium is the most important part to determine eye irritation, because it is located on the outermost layer of the cornea, which protects the underlying tissue by excluding foreign material. There are several corneal models used to assess eye irritation including EpiOcular™, SkinEthic HCE, the Labcyte Cornea model and MCTT HCE™. In particular, with regard to the reconstructed corneal tissue, the cells form a stratified and well organized epithelium that is structurally, morphologically and functionally similar to the human cornea with the presence of basal, wing and mucosal cells.[34] These models are used to study drug delivery, as they represent a metabolically active tissue with the presence of tight junction, characteristics of the human corneal epithelium. In addition, it has been shown that this type of tissue can be stimulated for the release of cytokines characteristic of an inflammatory state.

3. Computational aspects for the ocular pharmacology and toxicology

Along to *in vitro* models, for characterizing the pharmacological profile of possible drug candidates for treating ocular diseases, nowadays are growing different *in silico* approaches. These computational methods could be extremely useful for assessing the performance of a given drug candidates saving money and time with respect to the drug discovery pipeline. In particular, computational pharmacology and toxicology represent a specified field of research comprehending *in silico* approaches for predicting, modelling, and explaining pharmacological effects and toxicological mechanisms at the molecular level. Several researches have described the usefulness of *in silico* techniques for rapidly determining pivotal physico-chemical properties in order to optimize drug candidates (e.g., molecular weight, polarity, and lipophilicity). This computational evaluation is crucial for reducing off-target effects and therefore the total number of animals required for the *in vivo* test. Computational pharmacology and toxicology take advantage from numerous scientific disciplines and usually includes the application of *in silico* and statistical approaches for evaluating the bioactive profile of molecules for which a specific pharmacological or toxicological effect is not known, starting from a group of molecules for which the mentioned effect have been proven (training set).[35-38]

Accordingly, *in silico* strategies used for assessing the profile of compounds are mainly based on structure–activity relationship (SAR) and quantitative SAR (QSAR). In fact, most categories of computational methods in pharmacology and toxicology are based on the similarity principle: the hypothesis that compounds possessing a structural similarity could show comparable pharmacological or toxicological profiles. Numerous *in silico* techniques are commonly used for predicting both on- and off-target pharmacology of potential drug candidates.[39-40] Moreover, the use of computational approaches is decisive to limit animal testing also for the evaluation of potential ocular drugs and their possible toxicity. Currently, as above mentioned, the general evaluation of potential drugs is largely based on animal testing. In this contest, the valuable advances in

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computational models are facilitating to amend this standard. First of all, the regulatory agencies are encouraging the usage of *in silico* toxicology models for accomplishing the growing public request in order to improve animal welfare. This latter has convinced the governmental organizations to boost the reduction of animals used in *in vivo* tests encouraging alternative procedures for evaluating promising potential drug candidates. This exigence is well enclosed in the 3Rs principle.[41] Accordingly, the computational tools employed to characterize a given set of compounds, are almost without cost and they are applicable for virtual molecules before their synthesis, limiting the use of animal in preclinical development, testing only the most promising computational hits. Classical QSAR analysis for determining potential pharmacological profiles have been amended for predicting general toxicity and ocular toxicity as well as the side-effects of drugs, developing quantitative structure–toxicity relationship (QSTR) models.[42] This approach is widely used for generating models in order to computationally assess the potential toxicity of chemical entities. In QSAR approaches, the quality of the developed models is dependent on the chemical/molecular descriptors and the modelling strategies that are used. For example, early efforts about the QSAR modelling for predicting ocular toxicity were founded on the simple linear regression technique and empirical descriptors such as the physico-chemical properties.[43-44] This kind of models are surely easy to explain and implement due to their simplicity, but their efficacy is restricted to molecules that are extremely similar to the molecules included in the training set. Later, more complex modelling strategies and descriptors have also been applied in this field of research. For example, it is possible to use membrane-simulated models for studying ocular toxicity, identifying a group of descriptors that appropriately correlate to the cornea permeability. The individuation of appropriate descriptors, as in the mentioned case, were also used for developing eye irritation models.[45]

Furthermore, because only one type of descriptor and one modeling approach were used in most of the existent computational approaches, as the works centered on the Draize test data, the developed models for predicting ocular toxicity suffers from a difficult to predict toxicity for different structural unrelated chemical entities. This drawback is partially overcome by using improved QSAR method such as combinatorial QSAR (combi-QSAR) approach.[46] This technique relies on the use of numerous diverse combinations of many chemical descriptors and modelling strategies. By this method is highlighted that the improvement of the number of descriptors could be crucial for developing effective predictive models. Furthermore, combi-QSAR models can culminate in a consensus model (i.e., averaging of the results of all individual) in order to improve predictivity and coverage.[47] The main drawback of these model is surely the lack of sensitivity and/or specificity combined with an inability to predict the exposure to a given drug that would elicit the adverse effects, making these models needing of some improvements for their use as part of drug development trajectory. In general, these issues could be overcome by Machine Learning (ML)/Deep Learning (DL) approaches,[48] but actually regarding the ophthalmology, DL has displayed clinically satisfactory diagnostic performance, but only for detecting various retinal pathologies. In fact, DL in ocular imaging may be employed in combination with telemedicine as a potential solution for screening, diagnosing and monitoring main eye disorders (e.g. age-related macular degeneration, glaucoma, diabetic retinopathy, choroidal neovascularisation and other macular diseases).[49-50] Accordingly, only few examples of ML application to ocular pharmacology and toxicology are available and often referred to one ocular toxicity condition.[22, 51] Based on the previous discussion it is possible to predict, in the next years, a rapid growth of the computational approaches in this field aiming at reaching a significant improvement and robustness of *in silico* models regarding ocular pharmacology and toxicology.

4. Conclusion and Future Perspective

Thanks to the intense efforts that have recently been implemented by biomedical research in *in vitro* alternative methods, 3D corneal tissue models are becoming a real prospective of alternative

experimental models, in particular to Draize test which has been extremely criticized for ethical motivations. Based on the previous discussion it is possible to predict, in the next years, a rapid growth of the both 3D tissue model and computational approaches aiming at reaching a significant improvement and robustness of *in vitro* models regarding ocular pharmacology and toxicology. Furthermore, although not yet approved by OECD testing guidelines, in the last few years more innovative organoid (or organo-on-a-chip) *in vitro* models have been created. The development of this technology based on microfluidics closes the gap between *in vitro* and *in vivo* models by offering new approaches for pharmacological research. In fact, organs on chip can combine both preclinical models previously discussed, cultivating human cells in tissue-specific three-dimensional contexts. The advantage is that 3D cell culture models promote higher levels of cell differentiation and tissue organization than the usual 2D models.

Supplementary Materials: No Supplementary Materials are associated to this article.

Author Contributions: All authors have read and agree to the published version of the manuscript.

Funding: This work has been funded by Farmigea S.p.A. for the project entitled "Development of in vitro methods and pharmacological evaluation of innovative formulations for ocular diseases".

Acknowledgments: We are grateful to I-CARE EUROPE association for supporting this study with the project entitled "Reduction and Replacement" in ophthalmic disease.

Conflicts of Interest: S.O. and M.S. are employed of Farmigea S.p.A.. All the other authors declare no conflict of interest.

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 doi:10.20944/preprints202009.0756.v1