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REVIEW ARTICLE

# Animal models of Dry Eye Disease: Application to Drug Discovery

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ABSTRACT

Dry eye disease (DED), a multifactorial disorder of the ocular surface and tear film, affects 5-50% of the global population. Currently, no satisfactory treatments of DED exist. Ongoing efforts to identify novel therapeutic agents are handicapped by the limitations of its preclinical animal models, which to some extent reflect the pathophysiological complexities of DED.. A plethora of DED models employing multiple animal species (mice, rats, cats, rabbits, dogs, and non-human primates) has been reported, each aiming to capture components of DED that appear to determine its pathophysiology and response to novel treatments. Here, we review the main animal models of DED and attempt to place each in the context of drug discovery. We also discuss a nascent method for ex vivo culture of human conjunctival cells that may abbreviate early screening of candidate therapeutics. Despite the remaining challenges, there is justified optimism that with the contribution of these preclinical models, the development of an efficacious and safe treatment of DED will be forthcoming.

## Introduction

Dry eye disease (DED) is a multifactorial disorder of the ocular surface and tear film, characterized by disruption of the homeostasis of the tear film, leading to its instability and hyperosmolarity along with ocular surface inflammation [1-3]. The tear consists of three layers: the inner mucin coating produced by the conjunctiva's goblet cells; the aqueous component, the largest of the three, produced by the lacrimal glands; and the outer lipid layer secreted by the Meibomian glands [4].

Affecting 5% to 50% of the global population, DED is recognized as a growing public health issue, with its incidence progressively increasing [5]. The symptoms of DED are often a varying constellation of ocular pain, light sensitivity, dryness, irritation, and blurred vision [2]. Depending on its dominant etiology, DED has been classified as aqueous-deficient (decreased tear secretion), evaporative (increased tear evaporation) or mixed (a combination of the previous two) [2]. Despite enormous efforts over the years, no satisfactory treatment of DED has been developed [3]. Artificial tears provide transient symptomatic relief mainly in mild forms of DED, whereas pharmacological agents such as cyclosporin A and lifitegrast or punctal plugs are generally considered either ineffective or partially effective and are associated with side effects.

The therapeutic deficit in DED combined with its high prevalence and economic impact [6] have stimulated broad efforts to identify novel therapeutic agents. Current drug discovery requires informative preclinical models of the targeted disease, and this applies to DED as well. A plethora of DED models employing multiple animal species has been reported, each aiming to capture components of DED that appear to determine its pathophysiology and response to novel treatments [7]. That so many models have been developed underscores to some extent the fact that none of them fully meet the needs of the investigators, which is not surprising; it is almost axiomatic that no animal model can capture the full spectrum of a human disease.

Here, we review the main animal models of DED and present in more detail two models developed by us that we believe recapitulate key features of DED and promise to significantly contribute to DED drug discovery. Overall, mouse and rat models are most frequently used, followed by rabbit models, while those of cats and dogs are employed less frequently. As explained later on, models based on nonhuman primates are expensive, cumbersome and currently not highly informative. We also present a novel ex vivo method that may help accelerate drug discovery as it assesses drug responses in freshly cultured human corneal cells that maintain their pivotal in vivo features.

## Key Features of the DED Animal Models

As already alluded to, the ideal animal model of DED does not exist and, in all likelihood, will be impossible to ever develop. Thus, it is important to assess the existing animal models keeping in mind four critical features that could dictate an investigators' model selection for a specific project.

First, the *anatomical features* of the animal. The size of the animal per se determines how easy it is to handle it during experimentation. For example, mice are easier to handle than rabbits or dogs. More importantly, the *size of their eyes* can be a critical factor. In general, eyes of test animals closer in size to the human eye are easier to inject or to operate on or dissect to obtain tissues for analyses. Second, in certain circumstances the *molecular profile* of the eye can be important. As already pointed out [7], the expression of drug metabolizing enzymes in ocular tissues can impact drug discovery, as certain enzymes could inactivate a specific class of drugs, limiting the value of their study. Third, the *availability of species-specific reagents* heavily favors the mouse in all experimental studies that require molecular biology assays. Moreover, for studies that depend on specific genome parameters, the thoroughly studied murine genome and the many genetically engineered mouse models add further value to murine models. And fourth, some study animals can be more *costly* than others to acquire and maintain. An illustrative example is the striking cost differential between mice and non-human primates.

## Murine and Rat Models of DED

The murine models of DED, evolved over two decades, initially tried to recapitulate the features of Sjögren's syndrome, the prototypical autoimmune disease affecting the lacrimal glands. DED is a cardinal clinical manifestation of Sjögren's syndrome [8]. Sjögren's syndrome, affecting 0.5% to 1.0% of the population, is characterized by foci of lymphocyte-rich mononuclear cells infiltrating the lacrimal glands adjacent to blood vessels and the excretory ducts. These foci are comprised predominantly of T lymphocytes and culminate in sicca symptoms, i.e., dryness most often involving the eyes and mouth.

Many mouse models have been developed to replicate Sjögren's syndrome, but none of them capture all aspects of the condition. The *MRL/lpr*

*mice* develop dacryoadenitis with T-cell-derived inflammation (most of them are CD4 T cells) [9,10] starting at the age of 1 month and progressing rapidly, with the mice dying at 6 months. The mutation of the lpr gene alters the Fas protein, causing lymphoproliferation and elimination of activated T cells. The *non-obese diabetic (NOD) mouse* exhibits secondary autoimmune dacryoadenitis characterized by an infiltrate of CD4+Th1 cells [11-13]. At least six more murine models of Sjögren's syndrome have been reported to capture specific aspects of its pathophysiology (reviewed in [14]).

Pflugfelder's group developed an innovative mouse model of *desiccating DED* in which a constant low-humidity air flow aimed daily for 4 h at the face of the mice causes desiccating stress, leading to decreased tear secretion [15]. Combining the desiccating stress with muscarinic blockade produces severe DED simulating the clinical variant of aqueous-deficient DED [15-20]. The underlying immune mechanisms (CD3 (+) T-cell infiltration, higher Th17-cell activity, and Treg dysfunction) have been worked out, while a variation of this method can generate chronic DED [19,20].

*Surgical excision of the lacrimal glands* of mice predictably reduced (but did not eliminate) tear production leading to DED via an inflammatory infiltrate of the ocular surface [21,22]. It is important to note that the removal of both intraorbital and exorbital lacrimal glands was required to produce severe enough DED for this model to be useful. Their experience with mice is consistent with our own with rabbits (presented later) and suggests that the redundancy of the tear production system allows ample defense against immunological and environmental insults.

The development of rat models of DED proceeded in parallel with that of mouse models, no doubt prompted by the similar features of the two species, such as their easy handling and the low cost to acquire and house them. Being bigger than mice, rats have somewhat bigger eyes, but in practical terms this difference is not significant [23]. Several rat DED models have been inspired by their mouse counterparts (and vice versa) and are analogous to them, although experience with rat models of DED is often limited. Nevertheless, it is for their great similarities that mice and rats are reviewed here together.

The *computer and visual display terminal syndrome* is a corneal epithelial disorder caused by prolonged exposure to visual displays, a low humidity environment, and repetitive tasks [24,25]. Decreased blink frequency as a result of intense attention to the screen represents its key pathogenesis [26]. Of particular importance given the rising use of screens in daily life, this syndrome manifests as evaporative DED. A rat model simulating this syndrome has been established by housing rats under continuous low humidity airflow and placing them on a balance swing for 7.5 hours a day [27]. As a result, the rats display abnormal lacrimal gland morphology and impaired function with chronic reduction of tear secretion, and superficial punctuate keratopathy similar to that in humans.

The *scopolamine model* in rats, an aqueous-deficient DED model, mimics the scopolamine mouse model [28]. To induce this model, six-week-old female Lewis rats were given subcutaneously scopolamine for 28 days. This led to reduced tear production, tear film stability, and conjunctiva goblet cells, while corneal fluorescent staining increased indicating corneal damage [28]. The resulting symptoms make this model appropriate for the study of moderate DED.

One of the etiologies of DED is exposure to *environmental pollution*. Particulate matter can cause corneal inflammation with damage to corneal epithelial cells and reduced tear stability [29]. A rat model for this etiology was developed by exposing the rats to varying concentrations of a particulate matter aerosol for 5 hours daily for 14 days [30]. The aqueous tear volume decreased while the inflammatory index and damage to the corneal epithelial cells increased, all in a concentration-dependent manner.

## Cat and Dog Models of DED

Cats and dogs are closer to humans in terms of the anatomy of the eye compared to mice and rats. Of note, the use of dogs with spontaneous aqueous-deficient DED has contributed to major advancements in the treatment of DED in humans [31]. Specifically, the study of cyclosporine for the treatment of DED in dogs provided preclinical data for its use in humans and established the dog as an important spontaneous preclinical model of human ophthalmic disease [32,33]. Despite such illustrious beginnings, however, dogs (and cats) are not extensively used in the study of DED.

The eye of the cat is anatomically and physiologically similar to the human eye. However, cats are not widely used in DED studies because removal of the lacrimal gland does not result in significant changes in the ocular surface [33,34].

As pointed out by Hisey et al [31], dogs represent an appealing animal model of DED, its main advantages being that they exhibit a spontaneous

disease; the size of the eyelids and ocular surface of dogs is more similar to humans than that of rabbits and rodents; and collecting meibum from the eyelids and performing diagnostic tests are easier in dogs that all other animals except primates. Moreover, dogs and humans have similar physiological parameters, such as blink and tear turnover rates, and similar lipid composition of the meibum and ocular surface mucins, which stabilizes the tear film.

The *main lacrimal gland ablation model* is the second dog model that has been used with some frequency [35]. This model includes the bilateral removal of the orbital and nictitans lacrimal glands, which after 2 weeks induces DED, which lasts another 4 weeks. Tear production is reduced and clinical features, such as conjunctival hyperemia and tenacious discharge are present.

## Non-human Primate Models of DED

Monkeys have many features they could make them the ideal DED animal model. Anatomically, humans and monkeys are the closest, having globes of similar size, one main lacrimal gland, and lacking the nictitating membrane. Despite their anatomical similarity, it has proven challenging to develop an informative model for DED in primates. For example, when the lacrimal gland of primates was removed, tear secretion decreased but there was no damage to the ocular surface, rendering this model impractical [33,36].

*Desiccating stress-induced DED*. Researchers induced the model in rhesus monkeys, housed in an environment-controlled room with low humidity (15%) and constant airflow of 12 L/min [37]. Clinical symptoms of DED, reduced tear volume, disruption to tear film stability, and increased corneal epitheliopathy, were developed 2 weeks after desiccating stress was induced.

## Rabbit Models of DED

In our view, the rabbit could be the animal of choice to study various aspects of DED. It stands apart from the smaller rats and mice in terms of size and anatomy that allow their relatively easy handling. Moreover, rabbits have eyes of considerable size that permit comfortable surgical or other manipulations and simplify the performance of DED diagnostic tests. At the same time, while the size of their globe is not markedly smaller than that of dogs and non-human primates, their cost is significantly lower. An additional advantage of the rabbit is that its ocular expression of carboxylesterases is similar to that of the human, a crucial consideration for drugs that have carboxylic esters in their structure. Not surprisingly, there are over a dozen rabbit models of DED covering all pathophysiological aspects of this disease. Below, we summarize the majority of them, followed by a more detailed presentation of two models that we have recently reported, one a model of aqueous-deficient DED induced by the mitogen concanavalin A and the second a model of complete dacryoadenectomy.

*Aqueous-deficient DED* has been induced in rabbits using diverse means such as irradiation of lacrimal glands; administration to lacrimal glands of agents producing dacryoadenitis, e.g., lymphocytes and of an array of pharmacological agents such as atropine and benzalkonium chloride (reviewed in [38]). *Evaporative DED* has been induced by injecting *Mycobacterium tuberculosis* in Freud's adjuvant into the eyelids [39] or by closure of the Meibomian gland orifices by cauterization, which removes the outer lipid layer of the tears accelerating their evaporation [40]. Finally, combining elevated temperature (24°C/55% relative humidity) with keeping their eyes open for 1-3 h with an eyelid speculum causes *acute desiccative stress* that severely damages the cornea [41]. This model has been used, for example, to assess the contribution of desiccation to the pathophysiology of DED.

*Concanavalin A-induced model*. Concanavalin-A (Con-A) is a protein that has been used in experimental models of DED [42]. Nagelhout et al developed a model of lacrimal gland (LG) inflammation in rabbit models after a single injection of Con-A into the inferior LG. The injection technique was done without guidance by slightly retracting the lower eyelid and inserting a needle approximately 1 cm from the nasal canthus into the suborbital space to a depth of approximately 6 mm. Although the model induced a rapid onset of DED, which was responsive to treatment with dexamethasone, this method has not been broadly applied to drug development due to its inherent limitations including short duration of DED (1-2 weeks) and inconsistent animal responses.

Appreciating however, the merit of this method, our group developed three novel improvements to overcome its shortcomings: 1) The inferior lacrimal gland (LG) was injected using ultrasound guidance to ensure consistent Con-A delivery overcoming the physiologic variation among animals in size and location of the LG, 2) Development of a technique to also inject the superior orbital and palpebral portions of the LG thereby producing a consistent and uniform reduction of tear production by the entire LG apparatus. And 3) Use of weekly injections of Con-A to extend the duration of the

disease, as desired. In fact, several repeat injections of Con-A (usually 5 or 6) can convert the acute DED into chronic [43].

Con-A treated rabbits showed a strong inflammatory response in the LG with decreased tear break up time and Schirmer's tear test (STT) results while tear osmolarity values increased. Rose bengal staining also indicated compromised corneal and conjunctival epithelium [43].

With our novel improvements, this model became far more suitable for use in novel drug discovery studies. For example, this model was used to compare the efficacy of lifitegrast and cyclosporin to an experimental agent, phospho-sulindac (PS, OXT-328), chosen because of its anti-inflammatory properties, as seen in the treatment of arthritis in animal models [44,45]. PS formulated as nanoparticles and applied as eye drops three times a day for 20 days was effective in restoring the tear break up time, tear osmolarity, and tear lactoferrin levels indicating the suppression of DED in rabbits [46]. PS also performed better than the commercially available agents.

*Complete dacryoadenectomy model*. Complete dacryoadenectomy is the removal of the whole lacrimal gland apparatus. As previously performed dacryoadenectomy in rabbits only removed the inferior lacrimal LG. It was found however, that the partial dacryoadenectomy produced variable results, sometimes without significantly decreased tear production [7]. The variability in response occurs because the superior LG compensated for the lack of tear production following the removal of the inferior LG.

To address these shortcomings, we developed a surgical technique where both the inferior LG and the superior LG (both its orbital and palpebral components) were removed [47,48]. The orbital portion of the superior LG was removed via a transcranial approach through the top of the skull; the palpebral portion of the superior LG was removed via a transconjunctival approach through the everted upper eyelid. Lastly, the inferior LG was removed though a transcutaneous approach below the lower eyelid [48]. Nictitating membranes were removed prior to the removal of the LG in these protocols as well.

This model consistently removes all tear production from the orbital LGs. Determination of tear osmolarity, tear break up time, Schirmer's tear test, and rose bengal staining of the ocular surface showed that the complete dacryoadenectomy-induced DED resembles the clinical features of human DED [48]. Interestingly, removal of all orbital LG tissue did not completely obliterate tear production as measured by STT, indicating that the accessory LG in the conjunctival surface produced tears in an effort to preserve the ocular surface.

## DED Data Analysis Using Principal Component Analysis

Preclinical assessment of investigative drugs requires accurate analysis of efficacy. The diagnosis of DED in animals and humans rests on several parameters that often need to be evaluated together, a challenging task given that many parameters do not correlate. Principal component analysis (PCA) is a multivariate statistical technique that analyzes correlated variables and reduces data variability by expressing the results in a new set of independent variables called principal components [49]. PCA is increasingly used in biology and drug discovery [50].

We have evaluated whether PCA by "incorporating" the contribution of multiple DED test parameters could provide a better assessment of this disease compared to using individual clinical tests [43]. In our study, we applied PCA to compare normal and dry eyes from two different rabbit models, complete dacryoadenectomy and the Concanavalin A model, both described above. Tear breakup time, tear osmolarity, Schirmer's tear test, and rose bengal staining were the parameters used in this analysis. PCA defined objective variables (principal component scores), which could more effectively differentiate normal from DED than the individual test parameters. PCA provided a simpler, more comprehensive assessment of DED than the individual clinical test parameters, thus establishing the utility of PCA in this context.

This approach, easily applicable to studies of candidate therapeutics for DED, could prove very useful in the evaluation of drug efficacy in preclinical animal studies.

## Drug Screening Using Fresh Human Conjunctival Cells ex vivo

Drug development, resource- and time-intensive, extensively employs immortalized cell lines to screen candidate drugs for efficacy and safety. Unfortunately, their predictive value is low; e.g. [51,52]. While ex-vivo models more faithfully reproduce diseases, they are technically challenging to establish. Our group recently developed a simple method for ex-vivo culture of ocular surface cells and demonstrated its applicability to drug development [53].

In its essence, this method includes harvesting by impression cytology conjunctival cells, which are then grown on cellulose membrane filters. The two prominent features of this approach are 100% cell viability at 24 h and the intact expression of two thirds of 84 genes involved in ocular inflammation. In an exploratory study, human conjunctival cells cultured with this method were treated with a small molecule under development for the treatment of dry eye disease. We studied the presence of CXCL10, a chemokine elevated on the ocular surface and in the tear film in DED [54]. The cultured conjunctival cells displayed increased CXCL10 levels and the small molecule we tested suppressed CXCL10 levels [53]. Thus, human conjunctival cells cultured ex-vivo not only maintain their biological integrity, but they also respond to pharmacological agents.

This method, when fully validated, could rapidly screen candidate drugs during the early stages of the discovery phase. Advancing only promising candidates to the next steps can save time, resources, and effort, thereby accelerating the development of novel agents for the treatment of ocular surface diseases.

## Conclusion

The development of novel treatments for DED represents a clear and pressing unmet medical need that stems from the high (and increasing) prevalence of this disease [3,55]; its clinical impact, especially that of its more advanced forms; its cost, estimated to be $11,302 per patient and $55.4 billion to the US overall [56]; and the limitations of currently available treatments [2]. The animal models described above are an indispensable part of the development of new therapeutics for DED.

The utility of these models is hampered by two factors. First, DED is not a homogeneous disease. Its pathophysiology, still incompletely understood, indicates that its multiple etiologies can enter its "vicious cycle" [57] at various points leading to the diversity of its clinical presentations. Second, the inherent limitations of animal models of any human disease notwithstanding, the DED models to date probably do not capture all those elements of DED that are essential for its clinical manifestations. Thus, it may be difficult to either identify or evaluate druggable targets. Our transcriptomic analysis of DED using the Con-A model in rabbits [58], which revealed the unexpected complexity of DED, supports the notion that the task may be more demanding than initially thought.

Progress in this field is, however, palpable. It is reasonable to expect that more refined models will evolve from the current ones and that the application of sophisticated high-throughput methodologies and data analysis will generate a powerful combination in the evaluation of drug candidates. It is also reasonable to expect that at least some of these models might identify druggable targets, which in turn will stimulate the development of new drugs or repurposing of existing ones.

Finally, approaches like the ex vivo evaluation of drug candidates that may circumvent the lengthy, costly, and often cumbersome preclinical steps of drug development [59] hold promise. Their predictive ability, however, needs to be validated in order for them to be applicable. Nonetheless, this rather nascent effort should serve as a stimulus for further developments in this area, as their contribution may be great in a field where effort and time are paramount parameters of success.

## Conflict of interest

BR has an equity position in Medicon Pharmaceuticals and Apis Therapeutics. The remaining authors declare that they have no commercial or financial relationships to disclose.

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