Critical appraisal of alternative irritation models: three decades of testing ophthalmic pharmaceuticals

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Abstract

Background: Testing ocular tolerability of ocular pharmaceuticals is an essential regulatory requirement. The current approved reference model (gold standard) for ocular irritation testing is the Draize test. However this method is subjective and involves using live animals, hence the need to develop alternative in vitro and ex vivo testing strategies.

Source of data: Pubmed, Science Direct, Scopus, Google Scholar, Medline, Current Content, Web of Science and validation reports from international regulatory bodies; The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and European Centre for the Validation of Alternative Methods (ECVAM) were searched for in vitro alternatives.

Area of agreement: Whilst no single in vitro test can effectively replace the Draize eye irritation test, regulatory bodies and cosmetic/pharmaceutical industries agree that there is a need for in vitro alternatives with validated endpoints to evaluate pharmaceutical ingredients and finished eye products.

Area of controversy: There is no single in vitro test / assay that can predict the ocular irritation potential of mild to moderate test substances.

Area timely for developing research: This review provides a critical appraisal of the selected in vitro and ex vivo ocular toxicity models recommended by international regulatory bodies. These include cytotoxicity methods, biochemical systems and ex vivo assays. The latter are approved by ECVAM as in vitro alternatives for the well-known Draize test. Hen’s egg test-chorioallantoic...
membrane and the isolated rabbit eye test are also accepted by regulatory agencies in France, Germany, the Netherlands and the UK. A combination of ex vivo assays along with histological examination of excised bovine cornea can predict the conjunctival and corneal tolerability and cover a wider range of ocular pharmaceutical substances.

**Key words:** Draize test, ocular toxicity, irritation, cornea, hen’s egg chorioallantoic membrane, bovine cornea, opacity and permeability

**Introduction**

In recent decades, researchers testing ocular dosage forms have recorded toxicological signs of ocular tissues exposed to topically applied cosmetics and pharmaceuticals. Ocular tissues, such as the cornea and conjunctiva, are susceptible to injuries and adverse ocular effects, either from the administered drug or from pharmaceutical ingredients (excipients) used in formulating ophthalmic products.\(^1\)\(^,\)\(^2\) Antifungal agents, such as amphotericin B and ketoconazole, can cause corneal oedema and corneal abnormalities when administered topically.\(^3\) Excessive use of topical anaesthetics can produce corneal lesions and ulcers.\(^1\) Anti-inflammatory corticosteroids have been shown to retard epithelial corneal wound healing and induce glaucoma.\(^1\)\(^,\)\(^2\)\(^,\)\(^4\)

Ocular side effects due to excipients used in pharmaceutical products have been reported. Many surfactants have been studied as ocular penetration enhancers. Non-ionic surfactants including Span 20, Span 40, Span 85, Tween 20, Tween 40, Tween 81, Brij 35, Brij 58, Myrj 52 and Myrj 53 were tested for their ability to increase human corneal permeability of fluorescein.\(^5\) Benzalkonium chloride (BAC), a quaternary ammonium cationic surfactant, is a commonly used preservative in ophthalmic products. BAC has been reported to cause corneal opacification, a decrease in corneal epithelial microvilli, conjunctival hyperaemia (red eye) and delayed wound healing.\(^2\) The use of surfactants in ophthalmic formulations and ocular drug delivery is occasionally recommended to increase bioavailability.\(^6\)

A preliminary requirement of any ocular formulation is the lack of local adverse effects, e.g. burning, stinging and tearing. However, this is not always the case, especially with newly developed advanced drug delivery systems, e.g. micellar, reverse micellar, liquid crystalline, microemulsions, niosomes, liposomes, organogels and coarse emulsions. Assessment of the toxicity of ophthalmic formulations and the potential for ocular side effects represents an essential step in the development of new ocular formulations.\(^1\)\(^,\)\(^7\) On the regulatory side, there is relatively little guidance from the International Conference on Harmonisation (ICH) for non-clinical toxicity studies on ocular drugs, including those with novel additives.\(^8\)\(^,\)\(^9\)

European regulatory authorities recommend an in vivo ocular tolerance study (CPMMP/SWP/21/00), where a single dose (20–30 µl) of the test formulation is investigated in a small number of New Zealand white rabbits (one to three) for any ocular abnormalities that are recorded and scored.\(^9\)

The standard in vivo eye test (Draize eye test) has been around since 1940s.\(^10\) Accordingly, 100 µl of liquid or 100 mg of a solid test material are placed onto the lower conjunctival sac of the albino rabbit eye and the eyelids are closed for a fixed time period. Ocular responses are assessed by an observer at various time intervals for a period of up to 3 weeks after treatment. The contralateral eye is used as an untreated control. Three ocular tissues, namely the cornea, iris, and conjunctiva are scored for their irritation responses. A numerical score is given to different aspects of the irritation response observed. The maximum score is 110, with 80 points possible from the cornea, 10 from the iris and 20 from the conjunctiva. Because damage to the cornea is most critical, its contribution to the overall score is the greatest.

This methodology has been widely challenged as it lacks reproducibility.\(^11\) In addition, the use of sentient animals for ocular irritation testing has become a major target of criticism by animal welfare organizations. Thus, there has been an increased ethical and
scientific demand to use alternatives for ocular tolerability rather than testing on live animals.\textsuperscript{12} Different \textit{in vitro} and \textit{ex vivo} tests have been used for assessing the ocular toxicity of ophthalmic dosage forms and will be covered in this review; they are

(i) Cytotoxicity tests using cultured and isolated cells
(ii) Biochemical systems based on synthetic proteins (\textsuperscript{EYETEX\textsuperscript{®}}/\textsuperscript{IRRITECTION\textsuperscript{®}})
(iii) Red blood cell (RBC) haemolysis assay
(iv) Slug mucosal irritation (SMI) test
(v) Hen’s egg test-chorioallantoic membrane (HET-CAM)
(vi) Bovine corneal opacity and permeability (BCOP)
(vii) Combination of HET-CAM and BCOP
(viii) Histological documentation of treated bovine corneas

The most appropriate \textit{in vitro} toxicity tests should target the same endpoints as the Draize rabbit eye test in a more objective manner.\textsuperscript{13} Corneal opacity has been simulated by assays that measure protein coagulation, for example the \textsuperscript{IRRITECTION\textsuperscript{®}} assay, the RBC lysis test (which can also measure protein denaturation) and the BCOP test. Inflammation is a complex process that involves, amongst other factors, production of mediators (prostaglandins, leukotrienes, cytokines, etc.), increased microvascular permeability and invasion of white blood cells. Such a complex cascade of events may be better modelled using organotypic tests (e.g. BCOP, HET-CAM). The European Centre of the Validation of Alternative Methods (ECVAM) and Scientific Advisory Committee (ESAC) announced the validation of BCOP and isolated chicken eye tests in 2007. The HET-CAM and isolated rabbit eye test are accepted by regulatory agencies in France, Germany, the Netherlands and the UK.

**Cytotoxicity tests using cultured and isolated cells**

There are a number of simple \textit{in vitro} cytotoxicity assays that measure cellular functions such as growth, metabolic viability and plasma membrane integrity. The recommendation from ECVAM has been for the use of four cytotoxicity assays: the Cytosensor Microphysiometer (CM), neutral red uptake, RBC haemolysis and fluorescein leakage (FL).\textsuperscript{14} These \textit{in vitro} tests are recommended for drugs ranked at the bottom of the list of potential irritants. For drugs that are likely to be strong irritants or corrosives, organotypic tests (BCOP, HET-CAM) have been recommended. Hence, some assays have become more popular, others less so, as validation and testing help better identify the most appropriate replacements for the Draize test.

An early comparison of \textit{in vitro} test results with \textit{in vivo} scores obtained using the Draize test was conducted utilizing results from a group of seven \textit{in vitro} assays.\textsuperscript{15} The most promising correlation with \textit{in vivo} data was observed with the BCOP, HET-CAM and the former \textsuperscript{EYETEX\textsuperscript{®}} assay (currently called \textsuperscript{IRRITECTION\textsuperscript{®}}). \textit{In vitro} tests that correlated less satisfactorily with the \textit{in vivo} data were the TOPKAT comparison (\textit{in silico} prediction using structure-activity data), neutral red uptake (cytotoxicity), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in living dermal equivalent (cytotoxicity) and MICROTOX (cytotoxicity in bacteria) assays. To date, no single \textit{in vitro} test/assay has been fully accepted as an alternative to the Draize test by the main regulatory bodies.\textsuperscript{14}

**CM**

The CM test is a potentiometric assay that measures changes in extracellular pH, due to the release of acid equivalents from cells, as an index of metabolic rate, e.g. glucose utilization. The CM test has been accepted by ECVAM for the detection of non-irritants from amongst water-soluble surfactants and water-soluble surfactant-containing mixtures.\textsuperscript{16} The Organisation for Economic Co-operation and Development (OECD) has issued a Test Guideline describing in detail the CM test, its development from INVITTOX protocol No 102 and evaluation by ECVAM, ICCVAM, amongst others. Briefly, mouse L929 fibroblasts are cultured on a porous membrane/electrode and the release of acids is measured by a potentiometer. Negative control, positive control (sodium lauryl sulphate) and test substances are applied. The MRD\textsubscript{50} values (the dose of the test material that induces a
50% decrease in metabolic rate relative to a negative control) of these chemicals are calculated and compared against cut-off values of MRD50 for non-irritants, mild/moderate irritants and strong irritants. The CM test is unique in that it measures ‘extracellular acidification’ (‘change in pH’) as a proxy of intracellular metabolism. Other aspects of cell metabolism may also be measured using older methods such as amino acid uptake for protein synthesis, or 5[H]-uridine incorporation for DNA production.

RBC haemolysis

RBCs are readily available from the local abattoir, no ethics application is required and their handling does not require cell culture facilities. The RBC assay has been used as a screening test in the soap and detergent industry. The principle of the test method is based on the observation that certain classes of chemical irritants may cause damage to cell membranes and/or cause denaturation of membrane proteins and other cellular proteins. Such events can be correlated with the initial inflammatory response in tissue irritation and with changes in protein conformation, such as those that occur in opacification of the cornea. The biological endpoints of cell lysis and protein denaturation have been used singly or in combination to predict the ocular irritation of certain classes of chemicals (mainly surfactants).

The RBC assay is based on testing the membranolytic and protein denaturation activity of test substances employing mammalian erythrocytes isolated from fresh blood. The percentage release of the native dye oxyhaemoglobin (HbO2) as a result of lysis of fresh blood cells is measured using UV-Vis spectrophotometry (Fig. 1). The concentration of a test substance that induces 50% lysis of the RBCs is an endpoint (H50%) for release of haemoglobin and cell lysis. Another endpoint is spectrophotometric measuring of denatured oxyhaemoglobin due to interaction with the test substance as an indicator of protein denaturation.

![Fig. 1](https://academic.oup.com/bmb/article-abstract/113/1/59/284764) Percentage release of HbO2 due to lysis of fresh blood cells in response to a pharmaceutical excipient (cycloextrin). The response (increased optical density/colour intensity with increased concentration) is quantified using UV-Vis spectrophotometry at 540 nm (taken from in house developed RBC assay; unpublished data).
The RBC assay has been used to test potential pharmaceutical excipients such as cyclodextrins (Fig. 1) to predict the cytotoxicity of mild to moderate irritants. The RBC assay has been undergoing validation by the Validation Management Group (VMG).20 The test is not effective for substances of limited aqueous solubility. Overall the test shows good intra- and inter-laboratory reproducibility (reliability) and represents one of the promising in vitro alternatives to in vivo testing with a good fit to prediction models for the assessment of acute ocular irritation caused by surfactants and their formulations.

**Fluorescein dye leakage**

The FL test is recommended by ECVAM (as above) and also by OECD, as an alternative to the Draize test. Madin Darby Canine Kidney (MDCK) epithelial cells are grown on a permeable Millicell™ insert.21 The MDCK monolayer is used to model the in vivo corneal epithelium. Test substances are then applied, including a positive control (Brij 35) to a corneal epithelium monolayer. The leakage of fluorescein from the apical to basolateral compartments (as result of damage to tight junctions for example) is measured. The extent of FL is calculated from the concentration of fluorescein in samples obtained from the basolateral compartment, quantified using a spectrofluorometer and compared with an appropriate blank, and a positive control.

**Neutral red uptake**

Neutral red uptake (NRU) measures the viability of cells through their ability to take up and concentrate a dye (neutral red) into lysosomes. Cells expend energy to protons pump into lysosomes and preserve the acidic interior of these organelles. The neutral red assay has many advantages, including greater sensitivity than the MTT assay.22 The NRU may also be followed by a sulforhodamine B (SRB) protein assay, thereby using two assays with different endpoints on the same plate and test chemicals. The NRU assay with the mouse balb/c 3T3 fibroblast cell line has been proposed as a replacement assay.23

**MTT assay**

The MTT assay is one of the best known in vitro cellular viability tests. The endpoint is the function of a mitochondrial enzyme (succinate dehydrogenase) that converts MTT (a yellow substrate) into an insoluble purple formazan product. Reduction in mitochondrial function, e.g. after a toxic effect (of drug or excipient), reduces the intensity of the MTT product, compared with control cells. Newer substrates, with better sensitivity, (e.g. XTT) or other advantages in assay throughput (MTS, WST-1) have been introduced. The MTT assay does not currently feature in recommendations by ECVAM or OECD. However, the short-time exposure (STE) test uses the MTT assay in CCL-60 rabbit corneal epithelium cells,24 as does the EpiOcular™ and SkinEthic™ 3D models (see below). The STE is under consideration by the OECD for the identification of category 1 chemicals.15

**Cell growth assays**

Cell growth/number or cell proliferation may be monitored by a host of assays, some of the simplest of which measure cellular protein by traditional assays (Lowry, Crystal Violet and Coomasie Blue). The newer SRB protein assay is more sensitive than other protein assays using visible dyes and is efficient in terms of using low-cost chemicals and basic equipment.25 Cell growth assays are extensively used in carcinogenic and anti-cancer drug testing. However, the cornea of the eye is fully differentiated in adults with no further cell growth (unless injured), so cell growth assays are of limited value. Whilst animal epithelial cells lines serve as good models for human epithelium,26 there is a greater move to develop human cell lines that grow in a manner more akin to what is found in the physiological conditions.27

**3D cell cultures**

Immortalized cells, or continuous cell lines, are extremely useful in toxicity testing, whether for ocular irritation, dermal irritation or for testing putative anti-proliferative/anti-cancer drugs. Many of the assays discussed above are performed using epithelial, or
non-epithelial, cells cultured on an impermeable (often plastic) growth matrix. Epithelial cells are distinctive in having a basolateral membrane and an apical membrane, with different ion channels, transporters and receptors, which are critical for the transport of ions and other molecules. Cells that are allowed to grow and polarize on a permeable membrane (such as a Transwell™ or Millicell™) form tight junctions and desmosomes between cells and create the barrier function of an intact epithelium.

New developments in cell culture use permeable supports coated with appropriate in vivo proteins for attachment and allow cells to grow as a tissue with a 3D architecture. The 3D Vitrigel™ assay uses human corneal epithelial (HCE-T) cells grown in a collagen matrix to represent connective tissue. Transepithelial electrical resistance (TEER) measurements are taken using a handheld voltmeter, or when the support is mounted in an Ussing chamber. Positive controls are drugs known to induce damage to the monolayer and reduce TEER, or to increase FL. The EpiOcular™ culture model features human keratinocytes grown on a permeable support. An air–liquid interface is adopted so that the cells form many layers, in common with the corneal epithelium. Toxicity testing is by the MTT assay. The SkinEthic™ model uses reconstituted human corneal epithelium. There is no air–liquid interface, so the cell model is akin to the corneal mucosa. Cellular viability is measured by the MTT assay. Histological quantification is also possible, as is the detection of lactate dehydrogenase (LDH, a large intracellular enzyme) or cytokine release. For example, HCE-T cells were frozen, cut into 5 μm sections using a Cryostat, stained with haematoxylin and eosin (H&E) and probed with antibodies for ZO-1 (tight junctions). Visualization of the 3D cellular structures after exposure to test chemicals allowed a toxicological assessment of erosion of cells and disruption to tight junctions. All three models are under validation by ECVAM.

Biochemical systems based on synthetic proteins

The interaction of a test material with biological molecules, such as lipids and proteins, and the subsequent changes have been shown to contribute to irritant effects. The IRRITECTION® assay (In Vitro International, Irvine, USA) is the updated protocol of the EYETEX® assay. IRRITECTION® is a commercial test kit that quantifies the irritation potential of a test material based on interaction with a synthetic protein matrix. Small quantities of test sample when presented to the IRRITECTION® reagent produce turbidity, which is directly proportional to the irritation potential of the sample. This turbidity is produced by changes in conformation and degree of hydration of the IRRITECTION® reagent proteins which mimics the response of the proteins in the cornea during injury or in response to ocular irritants. Turbidity is compared with that produced by eye irritant standards of known Draize scores. The Draize equivalent score is then determined from a calibration curve.

Despite the encouraging testing results obtained with some hydro-alcoholic formulations tested using this methodology, EYETEX® has shown poor correlation with in vivo testing. For example, the validity of the EYETEX® for the assessment of surfactant-based products was reported to be questionable and contradictory. In an investigation of the eye irritation potential of cosmetic product formulations, an overall testing error of 20% comprising 18% overestimation and 2% underestimation was reported and was associated with ethoxylated and propoxylated surfactants or alcohol-based formulations. The false results were attributed to basic physico-chemical incompatibilities of the test material with the EYETEX® reagent system. The current IRRITECTION® assay is a new version of the former EYETEX®, which has numerous assay protocols leading sometimes to inconsistent results. There remains a need for more data on the validity of this test before it could be considered by regulatory agencies. Furthermore, the IRRITECTION® test kit is relatively expensive, compared with other in vitro tests employing readily available excised tissues.

Slug mucosal irritation assay

The slug mucosal irritation (SMI) assay was developed to predict the mucosal irritation of pharmaceutical ingredients and formulations but it is yet to be
approved by regulatory agencies. It utilizes the terrestrial slug *Arion lusitanicus.* Test substances are applied onto the slug body (shell-less gastropods) and the induced mucosal secretions are measured against positive and negative controls, then expressed as percentage of body weight to interpret the irritation potential of the test substance. A test substance resulting in a total mucus production (MP) <3% is classified as a non-irritant, 3–8% MP is a slight irritant, 8–15% MP is a moderate irritant and >15% MP is a severe irritant.

In another study, tissue damage was assessed by measuring the proteins and enzyme released from the mucosal surface. Mild irritants result in production of mucus with only limited effect on proteins and enzymes. The SMI assay has been used successfully to predict ocular irritation potential for cosmetic and pharmaceutical formulations. This test has been developed and assayed in Belgium; the Belgian *A. lusitanicus* was used as a test organism. The MP is species dependent; different species can generate different responses to test substances. The SMI assay should be optimized and validated, if other slug species are used.

**HET-CAM**

The CAM is the vascularized respiratory membrane that surrounds an embryonic bird developing in the egg. It is a complete tissue including veins, arteries and capillaries. The CAM has been recognized as a possible model for predicting the irritant effect of

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![HET-CAM vascular responses](image.png)

**Fig. 2** HET-CAM vascular responses used to score controls and test substances; (A) no response, (B) hyperaemia, (C) haemorrhage and (D) clotting, adopted from Abdelkader *et al.*
chemicals on the conjunctiva. A modification of the CAM test, called the HET-CAM has been reported. The HET-CAM is a rapid, sensitive and inexpensive test, whereby fresh fertilized hen’s eggs are incubated at 37°C ± 0.5 and relative humidity of 62.5 ± 7.5%. On Day 3 of incubation, the shell is broken and the egg is grown in a purpose-made chamber; the actual testing is undertaken on Days 9 or 10 of development. The test substance is instilled onto the membrane, and after 20 s the membrane is irrigated with water. The membrane is then scored for irritant effects including hyperaemia, haemorrhage and coagulation/clotting at 0.5, 2 and 5 min (Fig. 2).

The HET-CAM reduces animal suffering, is simpler, faster and less expensive than the Draize test and employs a less subjective and more reproducible scoring system. It has been successfully used to assess the irritation potential of promising pharmaceutical systems including microemulsion, liquid crystalline, o/w emulsion, niosomes, gels and more recently in situ gelling films/inserts. Accordingly, negative and positive controls comprised 1-cm Whatman filter paper discs. These were immersed in phosphate buffer saline (PBS) (negative control) and 1% w/v BAC solution in PBS (positive control) and placed onto the surface of CAM. Test ocular films were moistened by dipping into PBS and then placed on the surface of the CAM. Conventional inflammatory responses of hyperaemia, haemorrhage and clotting/coagulation of the CAM blood vessels and capillaries were recorded and scored over 5 min similar to the standard HET-CAM test.

Until recently, no single test has completely replaced the Draize eye irritation test. However, the HET-CAM test has been accepted by regulatory bodies in many European countries, e.g. The UK, France, Germany and the Netherlands to be a full replacement for animal testing for severe irritants. Conversely, the HET-CAM has limitations for testing water-insoluble substances and substances that stain and obscure the visibility of the CAM.

**Isolated organs**

Isolated, enucleated eyes (IEEs) from bovine, porcine, chicken and rabbit were used as potential models for alternative tests to Draize. The results obtained from the IEEs test correlated well with in vivo data. IEEs do not require the use of live animals but they use animal by-products including redundant animal tissues for other experiments. More advantageously, IEE tests do not require ethics approval. The main argument against the acceptance of isolated rabbit eyes is their limited supply from domestic sources. Therefore, laboratory rabbits are still needed as eye donors.

The BCOP assay is a good example on IEEs assay. The BCOP assay involves an assessment of

![Fig. 3 Corneal manifestations used to score the test substances in the BCOP test; unstained (left), fluorescein-stained (right), reused with permission from Abdelkader et al.](https://academic.oup.com/bmb/article-abstract/113/1/59/284764)
corneal opacity and disruption of the corneal barrier (as assessed by the passage of a fluorescent dye) after exposure to the test material (Fig. 3). Despite the fact that corneal injury represents only one endpoint of in vivo eye irritation testing, damage to the cornea is so critical that it contributes 80 out of the total 110 points allocated in the Draize test. The BCOP assay is used across the cosmetic and pharmaceutical industries to evaluate the ocular irritation potential of surfactants, pharmaceutical intermediates and finished products.\textsuperscript{29,44,48}

The scientific Advisory Committee of the European Centre for the Validation of Alternative Methods (ECVAM) announced the validation of two in vitro assays: the BCOP and the isolated Chicken Eye (ICE) test. These two tests are an acceptable replacement of the Draize test for severely irritant substances; for mild irritants, animals are still required.\textsuperscript{20,31}

Combined HET-CAM and BCOP tests

There is a general agreement that the HET-CAM test serves as a validated ocular model for conjunctival irritation testing, because it responds to irritant substances with an inflammatory reaction similar to that produced by conjunctival tissue.\textsuperscript{20,39,49} However, corneal transparency is critical for normal vision.\textsuperscript{50} Consideration must be given to the integrity of the cornea when using the developed ocular formulation, hence, the drive to establish the safety of the test material on both the cornea and the conjunctiva.\textsuperscript{51} Combined isolated enucleated eyes and HET-CAM tests were developed, and these combined tests were simultaneously compared with the Draize test. A good correlation was obtained with a broad range of chemical substances.\textsuperscript{51} Conjunctival and corneal irritation of ocular niosomes and discomes and their

Fig. 4 Light micrographs of H&E stained bovine corneas showing full-depth normal corneal layers at two different magnifications (A and B); severely injured cornea with epithelial detachment and stromal vacuolization (C) and delayed coagulation and chromatin formation (D). Micrographs are for in house developed sections (unpublished data).
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ingredients were successfully evaluated by combined HET-CAM and BCOP assays.38

**Histopathological documentation of the bovine corneas post-BCOP testing**

The main limitation of the BCOP test is that it is not sufficiently sensitive for discriminating between mild to moderate irritants. Further, predicting ocular irritation using opacity and permeability endpoints is challenging when the test substances produce a delayed reaction by interacting with nucleic acids and mitochondrial proteins, instead of immediate loss of epithelial integrity. Histological examination of the cornea after treatment with test substances (Fig. 4) can provide a more comprehensive assessment of the depth of injury and cellular damage to the three principle layers of the cornea.52,53 Moreover, histological examinations can be used objectively to quantify changes in stromal thickness, stromal vacuolization, nuclei coagulation and delayed chromatin formation (Fig. 4).38 Irritant-induced corneal opacity results from disruption of the well-ordered collagen fibres matrix of the stroma and is always associated with stromal oedema. Measuring the stromal thickness has been employed to serve as a quantitative measure of the degree of corneal opacity.38

Epithelial defects and stromal oedema are two common causes of ocular irritation after topical application of test substances.54 Cell loss, vacuolization (presence of vacuoles or holes), pyknosis (nuclei coagulation), saponification (due to the effect of alkali) and separation of cells from Bowman’s membrane are characteristic lesions observed in the epithelium in response to physically or chemically induced trauma. Histological scores should be correlated with opacity and permeability scores obtained using the BCOP test, preferably in conjunction with HET-CAM test results. It is accepted that the greater the stromal oedema, the greater the corneal opacity found.50 A summary of all the aforementioned tests including their endpoints, regulatory status and chemical classes/formulations tested are summarized in Table 1.

**Conclusion**

Three decades of research has failed to yield an acceptable alternative to the standard Draize eye test. However, many in vitro alternatives to the standard rabbit (Draize) test have been employed by formulation scientists and pharmaceutical/cosmetic companies for screening new eye products. These tests and assays could alleviate animal suffering by employing immortal cell lines, non-human blood or excised tissues from local abattoirs, and are more objective endpoints compared with the Draize test. Assessment of ocular damage from cell lysis, protein denaturation, corrosion, saponification and cytotoxicity for a wide range of chemical substances has been attempted using these in vitro alternatives. However, none of these tests can effectively monitor corneal innervation or corneal sensitivity, nor can they discriminate between substances that cause mild to moderate irritation, or between substances that exert a delayed toxic effect at the sub-cellular level. Combining histological examination with conjunctival responses from HET-CAM, and corneal effects from the BCOP assay, seems the most logical approach for preliminary in vitro ocular irritation testing.

**Conflict of Interest statement**

The authors report no conflict of interest.

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