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Review Article

Alternatives to the Draize Eye Test

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ABSTRACT

Industry and regulatory bodies responsible for public health are actively assessing animal free tests to reduce the requirement for Draize testing. Draize rabbit eye irritation test developed in the 1940's is even today the only eye toxicity test officially accepted in the OECD countries for regulatory purposes in the classification of slightly and moderately irritating chemicals. The Draize test has been widely criticized for both scientific and ethical reasons, and alternatives have been investigated for several decades. Therefore in an attempt to minimize this conflict alternative methods have been investigated. This article presents those alternative methods that are currently the most developed and the most widely used.

Keywords: Draize eye test, eye irritation, ocular toxicity, alternatives

INTRODUCTION

The requirement to evaluate irritation properties of chemicals and consumer products that might come into contact with human eyes is responsibility of the chemical and cosmetics industry for the safety of their products, so they are interested in seeking strategies to guarantee a maximum of information valuable for the assessment of local compatibility. As such, the evaluation of eye irritation potential for a cosmetic product and its ingredients is essential to provide reassurance that a product is safe for consumers to use through intended and foreseeable uses and accidental exposures to the eye.[1] The Draize rabbit test[2], developed in the 1940's, is the only eye toxicity test officially accepted in the Organization for Economic Co-operation and Development (OECD) Guidelines[3] for regulatory purposes in the classification of slightly and moderately irritating chemicals. According to the OECD Guidelines for the Testing of Chemicals (OECD, 1987), a standardized animal test on rabbits' eyes was defined and is obligatory for the registration of new chemicals on the market. The in vivo rabbit test only needs to be performed as a last step, when safety assessments in all the other tiers by relevant in vitro tests have produced negative results. A variety of different scoring systems assessing the extent of injury to the corneal, the iridial and the conjunctival compartments of the eye are currently applied in different regulations ranging from the single tissue scores to the average weighed sum scores of all the tissues. The Draize eye test is the most widely criticized single toxicity test, and it has been estimated that more effort has been focused on finding alternatives to the Draize eye test than on all the other acute in vivo toxicity tests combined.[4] The most recent validation studies have shown that no present single test,

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combination of tests, or testing strategy of in vitro alternative methods is capable of replacing the Draize eye test completely[5]

The necessity to establish and to accept proper in vitro methods is challenged by the chemical and cosmetics industry and by new expected limitations in performing animal tests in the future. One of the most impressive examples of expected limitations is the Sixth Amendment of the EU Cosmetics Directives, in which it was planned to ban cosmetic products containing ingredients tested in animals from the beginning of July 2000. During the last few years a variety of in vitro methods have been published, especially to predict eye irritation potential.[6][7]

THE DRAIZE EYE IRRITATION TEST [2][8]

The Draize Test is an acute toxicity test devised in 1944 by Food and Drug Administration (FDA) toxicologists John H. Draize and Jacob M. Spines. The current Draize eye irritation test evaluates the changes observed in three tissues of the eye: the cornea, the conjunctiva, and the iris.[2] Albino rabbit (e.g. New Zealand White rabbit) is the usual test species. A group of 3-6 animals is normally used. In the original Draize test, the lower eyelid is pulled away from the eyeball, and, depending on the test material (liquid, ointment, paste, or solid), 0.1 ml or 0.1 g of the test compound is installed in the conjunctival cul-de-sac. The materials can also be placed directly onto the cornea. The other eye is left untreated or treated with the vehicle or excipient. A topical anesthetic drug is sometimes instilled before the test agent to avoid unnecessary discomfort. A washing procedure may also be included. The evaluations of ocular lesions are generally made at 1, 4, 24, 48, and 72 hours after exposure, and, if needed, at 4, 7, and 21 days. Several grading systems have been proposed, but the original Draize scoring method remains widely used. The scoring method involves weighting and summing six components of the directly observable changes on the anterior segment of the eye, including the density and area of corneal opacification, the severity of iritis, conjunctival redness, edema, and discharge. An illustrated standard guide is used to score irritancy. The eye irritation potential is often summarized as the “Maximum Average Score” (MAS), which is obtained by averaging the weighted scores for individual animals at each time of observation (such as 4, 24 and 48 hours) and selecting the highest of these averages. In the original Draize test, the test scores can range from 0 to 110 points. From the maximum score of 110 points, 80 points (73% of the total score) can result from the severity and size of the corneal opacity, 20 points from the conjunctival irritation, and 10 points from the severity of iritis. While the weight sum scores are still in use for the safety assessment of cosmetics, the OECD, the United Nations, together with other international regulatory authorities have recently agreed on a Globally Harmonized System of Classification and Labeling of Chemicals.[9] This is based on averaged single tissue observations, taking into account the reversibility of the observed effects. The Draize test has been criticized for many reasons, such as the dosing of test materials, the methods of exposure, the subjectivity of observations and scoring, the lack of discrimination of fine response differences, and the overestimation of the human response.[10-13] Also, the reproducibility of the Draize test has been found to be poor within and among laboratories.[14-17] The test volume used in the original Draize eye test (0.1 ml) exceeds about ten times the normal volume of fluid residing in the human eye. Despite the criticism in terms of its scientific validity and its ethical acceptability, the Draize eye test has remained until now the worldwide accepted official government-recognized procedure for

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predicting the potential irritant effect of chemicals in the eye, at least for moderately and slightly irritating chemicals. With the development of alternative non-animal methods to replace the Draize eye test, the data generated by the Draize test has also been used as a "gold standard", to which the performance of in vitro methods has been compared.[8]

ALTERNATIVES TO THE DRAIZE EYE TEST

Industry and regulatory bodies responsible for public health are actively assessing animal free tests to reduce the requirement for Draize testing. Success in developing and validating alternative tests to replace the Draize rabbit eye irritation test has remained elusive despite major efforts by the European Centre for the Validation of Alternatives (ECVAM), industry trade associations, individual companies and academia.[1] The interest in applying alternative methods is quite diverse. On the one hand there is an interest in mechanistic aspects involved in irritation processes, and on the other hand there are interests in establishing new methods to gain maximum information on the realistic hazard to human eyes. Impressed by the interest for increasing the reliability of in vitro irritation tests, and to discriminate between weak and severe irritants, a couple of alternatives to the Draize rabbit eye test have been established and integrated into in-house routine experimental work. In-house toxicological testing programs have included in vitro methods over more than a decade to analyse mechanisms of different irritation effects as well as for screening purposes.[7] Six major validation or evaluation studies took place between 1991 and 1997. The outcome of each of these studies was summarised by Balls *et al.* (1999).[18] No test was found capable of replacing the Draize rabbit eye test, but some of the assays showed considerable promise as screens for ocular irritancy. The main reason for this is the difficulty of comparing in vitro test results with historical animal data where the subjective scoring of tissue lesions in the eye in the Draize test provides variable estimates of eye irritancy. Other possible contributing reasons for the outcomes of recently completed validation studies are: a) the *in vitro* tests only partially modelled the complex *in vivo* eye irritation response, b) the protocols and PMs might have been insufficiently developed, and c) the choice of statistical approaches for analysing the data might not have been appropriate.[1][18] In 1998, the European Centre for the Validation of Alternative Methods (ECVAM) estimated that there are approximately 70 different alternative methods for the assessment of eye irritation potential. These methods can be divided into several categories, such as computer models based on structure-activity relationships and physicochemical parameters of the compound to be tested, tests with plants and microorganisms, cell culture methods, chorioallantoic membrane (CAM)- based assays in fertilized hen's eggs, organotypic models, and three-dimensional tissue culture models. Most of the proposed alternatives are good for classifying certain types of chemicals, though not all of the chemicals across the full range of eye irritancy. Moreover, a number of proposed alternative methods appear to be capable of distinguishing between non-irritants and severe irritants, but they are not especially good at classifying between materials of mild and moderate toxicity. The Draize eye irritation test is the most widely criticized toxicity test, and consequently, several national and international validation studies on alternatives for ocular toxicology have been organized.[8] The most developed and the most widely used alternatives are the red blood cell (RBC) assay, the agarose diffusion method, the hen's egg chorioallantoic membrane (HET-CAM) test, the chorioallantoic membrane trypan blue (CAM-TB) test, the organotypic bovine corneal opacity

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and permeability (BCOP) test, the isolated chicken eye (ICE) test, the isolated rabbit eye (IRE) test, and the EpiOcular™ tissue model.[5][8] Though not formally validated, the usefulness of these *in vitro/ex vivo* methods is well established within some national regulatory agencies and within industry for specific and limited purposes.[5] Confidence for such in-house use of some of the currently available *in vitro/ex vivo* methods is dependent on the availability of appropriate benchmarks, historical information on similar materials, an understanding of the limitations of the assay(s) and the technical expertise of the user. For these reasons, such use of *in vitro/ex vivo* methods is often company-specific and for cosmetic products manufacturers more often related to finished product testing.[1] It has been estimated that each year thousands of new products and materials are successfully tested worldwide in in vitro alternative studies, but only a small fraction of the results have been published.[19] Nevertheless, validation studies have not been able to establish this satisfactorily when in vitro test results have been compared to the historical Draize test data.[20] The main reason for this is the subjectivity of the Draize test, which provides variability in the estimation of eye irritation. It is now considered that a battery of in vitro tests reflecting the different mechanisms of eye irritation will be needed for the complete replacement of the multipurpose animal test.[21] However, in spite of the magnitude of the research focused on eye irritation, the mechanisms involved are not yet adequately understood.[8]

EPIOCULAR™ TISSUE MODEL[1]

MatTek's EpiOcular™ corneal model (MatTek, Ashland, MA, USA) consists of normal, human-derived epidermal keratinocytes that are cultured on specially prepared cell culture inserts using serum free medium, and that differentiate to form a multi-layered structure which closely parallels the corneal epithelium. EpiOcular has been utilized with several common tests of cytotoxicity and irritancy, primarily MTT but also IL-1a, PGE2, LDH, and sodium fluorescein permeability. Comparison with *in vivo* animal data has been carried out, by using the ET50 value (effective time of exposure to reduce tissue viability to 50%) determined by MTT assay. Using the variable of time rather than dose allows ingredients and formulations to be tested without dilution in medium. Thus both hydrophobic and hydrophilic materials may be tested. As a stratified epithelium, the EpiOcular construct is intended to model damage to the corneal epithelium and conjunctiva (with its very thin epithelium). Therefore, it can be used to resolve degrees of irritancy potential (cellular damage) in the moderate to very mild irritancy range (mild corneal and conjunctival irritation). The tissue construct is capable of also identifying high moderate and severe irritants by their very short ET50 values. However, based on the stromal changes associated with severe irritation, an epithelial construct would not be expected to provide the degree of resolution in the severe range that a full thickness cornea (e.g. ex vivo cornea) would provide. The assay is applicable to both hydrophilic and hydrophobic test materials (both formulations and ingredients). Either liquids or solids may be tested. Again, the best resolution has been obtained in the range of the mild to moderate irritants. The EpiOcular™ tissue is overly sensitive to the alcohol and esters group of chemicals.[18] In general, highly volatile liquids, organic solvents, and certain classes of reactive chemicals (e.g., peroxides) may not be appropriate for this model system.

ISOLATED CHICKEN EYE (ICE) TEST[22][23]

The ICE test method is an organotypic model that provides short-term maintenance of the

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chicken eye *in vitro*. In this test method, damage by the test chemical is assessed by determination of corneal swelling, opacity, and fluorescein retention. While the latter two parameters involve a qualitative assessment, analysis of corneal swelling provides for a quantitative assessment. The eyelids are carefully excised, taking care not to damage the cornea. Corneal integrity is quickly assessed with a drop of 2% (w/v) sodium fluorescein applied to the corneal surface for a few seconds, and then rinsed with isotonic saline. Fluorescein-treated eyes are then examined with a slit-lamp microscope to ensure that the cornea is undamaged (*i.e.,* fluorescein retention and corneal opacity scores ≤ 0.5). The eyeball is pulled from the orbit by holding the nictitating membrane firmly with surgical forceps, and the eye muscles are cut with a bent, blunt-tipped scissor. It is important to avoid causing corneal damage due to excessive pressure (*i.e.,* compression artifacts). When the eye is removed from the orbit, a visible portion of the optic nerve should be left attached. Once removed from the orbit, the eye is placed on an absorbent pad and the nictitating membrane and other connective tissue are cut away. The enucleated eye is mounted in a stainless steel clamp with the cornea positioned vertically. The clamp is then transferred to a chamber of the superfusion apparatus. The clamps should be positioned in the superfusion apparatus such that the entire cornea is supplied with the isotonic saline drip (3-4 drops per minute or 0.1 to 0.15 mL/min). The chambers of the superfusion apparatus should be temperature controlled at 32 ± 1.5°C. After being placed in the superfusion apparatus, the eyes are again examined with a slit-lamp microscope to ensure that they have not been damaged during the dissection procedure. Corneal thickness should also be measured at this time at the corneal apex using the depth measuring device on the slit-lamp microscope. Eyes with; (i), a fluorescein retention score of > 0.5; (ii) corneal opacity > 0.5; or, (iii), any additional signs of damage should be replaced. For eyes that are not rejected based on any of these criteria, individual eyes with a corneal thickness deviating more than 10% from the mean value for all eyes are to be rejected. Once all eyes have been examined and approved, the eyes are incubated for approximately 45 to 60 minutes to equilibrate them to the test system prior to dosing. Following the equilibration period, a zero reference measurement is recorded for corneal thickness and opacity to serve as a baseline (*i.e.,* time = 0). The fluorescein score determined at dissection is used as the baseline measurement for that endpoint. Immediately following the zero reference measurements, the eye (in its holder) is removed from the superfusion apparatus, placed in a horizontal position, and the test chemical is applied to the cornea. Liquid test chemicals are typically tested undiluted, but may be diluted if deemed necessary (*e.g.,* as part of the study design). The preferred solvent for diluted test chemicals is physiological saline. However, alternative solvents may also be used under controlled conditions, but the appropriateness of solvents other than physiological saline should be demonstrated. Liquid test chemicals are applied to the cornea such that the entire surface of the cornea is evenly covered with the test chemical; the standard volume is 0.03 mL. If possible, solid test chemicals should be ground as finely as possible in a mortar and pestle, or comparable grinding tool. The test chemical (liquid or solid) is applied for 10 seconds and then rinsed from the eye with isotonic saline (approximately 20 mL) at ambient temperature. Each measurement is either converted into a quantitative score used to calculate an overall Irritation Index, or assigned a qualitative categorization that is used to assign an *in vitro* ocular hazard classification, either as UN GHS

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Category 1 or as UN GHS non-classified. Either of these outcomes can then be used to predict the potential *in vivo* serious eye damage or no requirement for eye hazard classification of a test chemical. However, no classification can be given for chemicals not predicted as causing serious eye damage or as not classified with the ICE test method. The ICE test method is not recommended for the identification of test chemicals that should be classified as irritating to eyes (*i.e.*, UN GHS Category 2 or Category 2A) or test chemicals that should be classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category chemicals overclassifed as UN GHS Category 2, 2A or 2B.

AGAROSE DIFFUSION METHOD[24]

Of all the *in vitro* methods described to evaluate cytotoxicity, the agar diffusion is the only one mentioned in the official bibliography, having been described.[25] This same method, with the graduation of the American Pharmacopeia 31[26], was used in this study to evaluate the safety of cosmetics, aiming at its correlation with the ocular and cutaneous irritation *in vivo* method. This study was also performed with the intent to verify the relation between the origin of cell lines and the target tissue used in the *in vivo* test. The use of animals in tests which evaluate cutaneous and ocular irritation caused by cosmetics has been causing polemic. Thus this has become a crucial matter, especially in the European Community, whose population is strongly in favour of new initiatives and cruelty-free cosmetics.[27] Toxicological methods are important, among these, the agar diffusion method is one of the pioneers of *in vitro* test. Due to its reproducibility it was adopted by the International Organization for Standardization (ISO) and by the United States Pharmacopeia (USP), as an official method for evaluation of plastic and medical devices.[26][28] Some institutions recommend the use of any mammal cell line, others specify NCTC clone 929 cell line, adopted due to its stability and easy handling. The study adopted not only NCTC clone 929, but also FPC – IAL and SIRC cell lines. The strategy results from the analyses of the close correlation between ocular irritation and *in vitro* cytotoxicity tests using cornea cells, as well as the cutaneous irritation test using human skin fibroblast.[29][30]

ISOLATED RABBIT EYE (IRE) TEST[1]

The isolated rabbit eye test (IRE test)[31][32] determines the opacification of the cornea and the increase in corneal thickness (corneal swelling) after exposure to irritant substances. Whole eyeballs obtained by immediate dissection from humanely killed laboratory rabbits with healthy eyes are mounted and maintained in a vertical position in a so-called superfusion chamber with controlled temperature and humidity. Pre-warmed saline solution is applied drop by drop directly onto the cornea at regular intervals to keep it moist. Prior to treatment with test sample a visual check for opacity is carried out together with an evaluation of the penetration of fluorescein and the swelling of the cornea, and the damaged tissues are excluded. The eyeball is then either taken out of the chamber or left in situ (depending on the type of test material) and exposed to the test chemical; for example, 10 seconds for identification of severe irritants and 1 minute (or longer) for the ranking of less severely damaging materials/products.[33][34] After removal of the chemical, the eye is repositioned in the chamber and the cornea is examined for evidence of opacification along with measuring of corneal thickness. Further assessments are made at 30 min., 1, 2, 3, and 4 hours after dosing. A check on fluorescein penetration is carried out 30 min. and 4 hours

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after treatment. Scores for corneal opacity (similar to Draize scores) and fluorescein penetration are recorded (qualitative assessments). For each test sample the mean percentage of corneal swelling of three eyes is calculated and compared to an untreated control eye. The preparation and examination of histological sections of the treated corneas can be used to confirm the level and depth of corneal damage. Overall damage is assessed by means of a combination of the different parameters scored, depending on the nature of the effects observed and in-house classification systems may vary.[33] Chemical substances causing the cornea to swell by more than 15% have been considered to have the potential to cause severe irritation of the eye *in vivo*[35], but a more complex classification model combining opacity, corneal swelling and histological observations of the corneal epithelium has been published.[36][37] The test is probably suitable for testing most types of test material, particularly where severe effects are observed, although physical effects of solids, which may be seen *in vivo*, are not always apparent *in vitro* (where the material is static on the treated cornea). There was a wide range of chemistry in the data submitted to IRAG.[38] In the originating laboratory most experience and usefulness has been obtained with respect to alkaline materials[34] and surfactants.[36][37] The 10 second application time is most suitable for distinguishing severe eye irritants; longer application times (e.g., 1 minute) are more suitable for the ranking of less damaging materials. The IRE test primarily predicts corneal effects and does not provide information on the effects of materials on the conjunctiva of the eye or on the recovery of the cornea from damage (beyond a few hours), which might occur in the eye *in vivo.* Absence of damage therefore does not suggest that there would be no effects *in vivo*.

BOVINE CORNEA OPACITY TEST (BCOP)[1]

By using slaughterhouse material the BCOP assay avoids the keeping and killing of laboratory animals. Freshly isolated cornea is mounted horizontally in a holder which is placed inside a specially modified opacitometer. The mounted cornea divides the test chamber into two compartments with controlled temperature and the test compound is added to the compartment enclosing the epithelial surface of the cornea.[39][40] After measuring opacity, a fluorescein-containing solution is added to the epithelial side (i.e., the upper compartment) in order to determine the permeability of the cornea by assessment of the optical density (O.D.) of the medium in the lower compartment. The measured numerical values for opacity and permeability can be used to calculate a so-called in vitro score.[41][42] Classification of test materials can be done according to this score. Better prediction of certain chemical classes is obtained with the addition of histological evaluation of the corneas. The BCOP assay is amenable to testing a wide range of physical forms and solubility characteristics. It is well suited to identify substances moderately and severely irritating to the eye, but seems to be not as sensitive in distinguishing among mildly irritating materials when applying the standard protocol. Histology can be an aid in good labeling of mild irritants. There seems to be a tendency to underestimate the irritant potential of substances acting more pronounced on the iris or the conjunctivae.[43] Recently, the BCOP assay was included in a project which evaluated several *in vitro* methods by applying reference standards in order to gain wider acceptance of such assays in the regulatory context.[18]

HCE-T TISSUE CONSTRUCTS (GILLETTE)[1]

To develop a reproducible model of the human corneal surface, the Gillette model uses a transfected human corneal epithelial cell line

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(HCE-T)[44] cultured on collagen-membrane cell culture inserts which, at the air-liquid interface, stratify to form a four- to six-layer epithelium, known as the HCE-T model. Transepithelial permeability to sodium fluorescein (TEP) and transepithelial electrical resistance (TER) have been identified as physiologically relevant parameters for evaluating the barrier function of the corneal epithelium.[45][46] Cell viability can be determined by the MTT assay, and histomorphology can also be used as an endpoint. The assay focuses on liquid surfactant-containing formulations. Only limited data are available on other types of materials. Cationic surfactants, which precipitate proteins, tend to be under-predicted with the current prediction model.

HEN'S EGG CHORIOALLANTOIC MEMBRANE (HET-CAM) TEST

One of the most robust and successful assays for the evaluation of the local compatibility of raw materials as well as of final products seems to be the HET-CAM, the hen's egg-test on the chorioallantoic membrane of fertilized chicken eggs. The HET-CAM was adapted for a broad spectrum of chemicals and optimized to cover the whole range of degrees of irritation and physical appearance of different substances. During the last couple of years, in Germany as well as France, the HET-CAM has been officially accepted as a valid in vitro assay, at least for the prediction of severe irritants.[7] As with some other organotypic models, the HET-CAM test permits the identification of irritative reactions which appear to be similar to those which occur in the eye using the standard Draize rabbit eye test. In the HET-CAM test system, three reactions are determined, namely, haemorrhage, lysis and coagulation (sometimes also hyperemia is used as a parameter) of the chorio-allantoic membrane at the ninth day of embryonation when nerve tissue and pain perception have not yet developed. After placing the test sample directly onto the CAM, an evaluation of the above mentioned parameters over a 5-minute observation period takes place. The most widely used approach is the reaction time method, in which the time until the appearance of each of the three endpoints is determined. Another approach is the irritation threshold method, which determines the concentration of the test material at which effects on these parameters are first observed. Whereas these approaches are mainly used for transparent test materials, a third approach for non-transparent insoluble and solid materials can be used by exposing the CAM to test samples for a fixed time (e.g. 30 seconds or 5 minutes) and examination for a.m. endpoints after careful rinsing to remove the sample. Although different scoring systems have been developed, the original HET-CAM scale, a weighted scale in which coagulation is given a higher weight than haemorrhage and lysis, is still widely used. The majority of the validation studies carried out, showed a useful correlation between the HET-CAM test and the Draize rabbit eye test for the assessment of raw materials and cosmetic products. This in vivo versus in vitro correlation revealed good results in the area of mild and non-irritating test materials as well as for surfactants and surfactant-based formulations.[48][49]However, regression analysis showed linearity only in this lower range of irritancy but not over the whole range of Draize MAS scores.[49] The mathematical prediction model used in the COLIPA study also showed certain limitations.[48] Although the HET-CAM assay in principle is applicable to all types of chemicals regardless of their physico-chemical properties, measurements on solid and insoluble or sticky materials may cause problems in the reproducibility of test results while pigments and dyes may cause interference by staining the CAM.[49][47] Additionally, when alcohol/esters and surfactants were tested comparatively

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using HET-CAM and NRU, a good correlation was observed for surfactants but not for alcohols and esters.[18] Regarding the measured end-points in the in vivo eye irritation test, the HET-CAM assay should mimic the conjunctival response of the Draize rabbit eye test. Although the HET-CAM test is regarded to be an established and reliable test for screening purposes, a further potential limitation can be seen in the absence of the possibility to assess reversibility and/or irreversibility of effects. Severity of effects will be covered by the HET-CAM while for methodological reasons the recovery or persistence of effects is out of the scope of the various HET-CAM test protocols used today.[1]

CHORIOALLANTOIC MEMBRANE TRYPAN BLUE (CAM-TB) TEST

The chicken chorioallantoic membrane - trypan blue staining (CAM-TB) method was developed to provide an objective evaluation technique to overcome disadvantages arising from the lack of objectivity and quantitativeness associated with the HETCAM. This method was designed to examine the injurious effect of substances by measuring the amount of trypan blue adsorbed with the CAM as the endpoint of the assay. Trypan blue staining, widely used for measuring cell viability, detects destruction and denaturation of the membrane.[47][1] The chorioallantoic membrane–trypan blue staining assay (CAM–TBS) is used to evaluate the potential ocular irritation caused by liquid scintillation cocktails constituted by complex mixtures, including surfactants and other potential irritants. The harmful effect of these substances is determined by the amount of trypan blue adsorbed by the CAM.[50]

RED BLOOD CELL (RBC) ASSAY[1]

The red blood cell (lysis) test (or RBC test) is based on the (cytotoxic) potential of a chemical substance to disrupt cell membranes. Membrane damage is assessed by measuring photometrically the leakage of haemoglobin from freshly isolated red blood cells incubated with test materials under standard conditions.[6][51-58] Access to mammalian erythrocytes is easy (e.g. slaughterhouse material). Thus, the RBC test contributes to the reduction of animal numbers used for eye irritation testing. Haemolysis and the denaturation of oxyhaemoglobin (oxyHb) are used as toxicological endpoints in the assay, which is carried out in two steps. In a range finding experiment haemolysis and oxyhaemoglobin denaturation (i.e. the change of protein configuration) is determined by measuring the reduction in absorbance at 541 nm (i.e. the one absorption maximum of oxyhaemoglobin) occurring within 60 minutes after exposure to increasing concentrations of test substance. The aim is to find the concentration range where haemolysis occurs. All determinations are made relative to a control sample, in which all erythrocytes were lysed (i.e. defined as 100% haemolysis). The main study serves to establish a more accurate concentration-response curve for haemoglobin leakage in order to calculate the concentration which causes 50 per cent haemolysis (H50 value). OxyHb denaturation determined in the range-finding experiment is expressed as D-max, i.e. the maximum percentage of denaturation seen at any concentration tested, and D-low, i.e. the lowest concentration at which denaturation becomes greater than 10 per cent. The studies by Pape and co-workers[54-58] showed that any test substance causing haemolysis invariably produced some degree of eye irritation in the Draize rabbit eye test. The endpoints H50 and D-low were found to be inversely correlated with Draize MAS values whereas Dmax was positively correlated with the MAS. The ranking of compounds by both the haemolysis and the oxyhaemoglobin denaturation endpoints, in particular, when

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employing the lysis/denaturation ratio (L/D ratio), correlated significantly with in vivo eye irritation rankings. Most of the materials used in the studies to establish the RBC test have been surfactants; thus, at present, the assay is specific to this class of chemicals. So far, the RBC test has not been validated for any other classes of chemicals. Importantly, the RBC test can only be used for water-soluble and water-dispersible substances. Coloured substances can be measured using different test parameters for the membrane damage, such as the transmembrane ion exchange and highly acidic or alkaline solutions may also not be suitable[51] which is not of importance since the acidic and alkaline materials are known to be severe eye irritants.[1]

CONCLUSION

The level of irritability of several substances and products for human use has been under evaluation since the 1940s, through experiments which use laboratory animals. At present the only worldwide accepted method for regulatory purposes is Draize rabbit eye test (Draize, 1959) for moderately and slightly irritating chemicals. Severe procedures which affect the safety of animals result in criticism and have been discussed by non-governmental entities. In recent years because of its controversial nature, the use of the Draize test in the U.S. and Europe has declined and is sometimes modified so that anaesthetics are administered and lower doses of the test substances used. Chemicals already shown to have adverse effects in vitro are not currently used in a Draize test, thereby reducing the number and severity of tests carried out. Therefore in an attempt to minimize this conflict alternative methods have been investigated. Main difficulty in the development of alternatives to the Draize test have been a lack of high correlation between in vitro alternative test results and in vivo Draize test results. The HET-CAM was adapted for a broad spectrum of chemicals and optimized to cover the whole range of degrees of irritation and physical appearance of different substances. In France and Germany during the last couple of years, the HET-CAM has been officially accepted as a valid in vitro assay, at least for the prediction of severe irritants. CAM-TB is objective and allows the evaluation of opaque and coloured substances without interfering in the determination of irritancy. Despite these advantages, the method is not suitable for complex mixtures of products that induce ocular irritation in small quantities. Both the HET-CAM and CAM-TB methods may present alternative method of evaluation of eye irritation despite problems of interlaboratory reproducibility. The validation of reconstituted human corneal epithelium models should be expedited, so that the detection of lower-level irritancy potentials can be achieved without the need for animals The ICE test method is an in vitro test method that can be used, under certain circumstances and with specific limitations for eye hazard classification and labelling of chemicals. The IRE test is considered to be well suited to identify substances 'severely irritating to the eye' according to EU classification R 41. It is considered to be a valid test for the (pre)screening of severely irritating materials. Agar diffusion method, due to its reproducibility was adopted by the International Organization for Standardization (ISO) and by the United States Pharmacopeia (USP), as an official method for evaluation of plastic and medical devices. Mostly surfactants are used in the studies to establish the RBC. Therefore at present, the assay is specific to this class of chemicals. RBC test can only be used for water-soluble and water-dispersible substances.

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