

In vitro alternative methods of eye irritation tests

Part 1

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One component in the safety assessment of many types of products is the evaluation of their potential to cause eye irritation or corrosion.



Until recently, the Draize Rabbit Eye Irritation Test, developed in 1944, had been the standard method for evaluating the ocular irritation/corrosion potential of a substance.

However, the Draize Rabbit Eye Test has been increasingly criticised due to its lack of reproducibility, its overestimation of human responses, and the cruelty to animals that it involves.

The recently implemented 7th Amendment to the EU Cosmetics Directive and the EU REACH legislation have heightened the need for *in vitro* ocular test methods. Changes in legislation and increasing pressure from animal rights organizations gradually eliminate the use of methods for testing of cosmetics ingredients on animals.

The following systems can be used as partial or full replacements of animals in toxicology experiments:

- *in vitro* methods: cell cultures, reconstructed tissues, co-culture systems,
- *ex vivo* methods: isolated animal tissues and organs,
- *in silico* methods: computer simulations and mathematical models, QSAR's

It would be perfect to replace traditional animal-based test methods by *in vitro* alternative method, what will allow to keep the testing regime simple and economical, obviously assuming that new *in vitro* method will provide the data of equal or better quality than the traditional *in vivo* test.

It's not that simple.

In vitro tests had generally been designed to model only one or just a few ocular tissues, not the whole eye. This is very helpful in obtaining more detailed mechanistic information about the process of eye irritation. However, it then potentially force us to replace a single animal test with multiple *in vitro* tests. This is not necessarily undesirable. Using several *in vitro* assays accurately depicting the mechanical aspects of eye damage, probably will allow us to learn more about the actual risk of the use of chemicals by humans.

According to ECVAM [European Centre for the Validation of Alternative Methods] in the case of eye irritation it is generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to replace the Draize eye test to predict across the full range of irritation for different chemical classes. However, strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the Draize eye test.

Definition

Eye irritation is defined as the production of changes in the eye following the application of test substance to the anterior surface of the eye, which are fully reversible within 21 days of application.

Eye corrosion (serious eye damage) is defined as the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application.



Eye

In interpreting the results from any toxicologic study, there must be some basic knowledge of the organ system being studied - at the very least an understanding of its morphology, cellular constituents, and normal function - that allows one to determine whether an injury has occurred and what the consequences of that injury are.

The eye is a very intricate organ made up of multiple tissues, each of which responds differently to injury.

Perhaps the most important tissue is the cornea. The normally transparent cornea allows light to freely enter the eye and eventually be focused on the retina. If the cornea becomes cloudy (opaque) - as can happen after accidental exposure to strongly irritating chemicals - light can no longer pass easily into the eye and vision becomes impaired or even completely blocked. Although the eyelids offer the cornea some protection, it is still very susceptible to injury.

About 80 % of the cornea's structure is the stroma - a regular array of macromolecules through which light can easily pass as a consequence of the stroma's high degree of order and exact level of hydration.

Maintenance of this very important hydration level (75-80% water) is the responsibility of two active cell layers:

- a single-cell-thick endothelium covering the inside surface of the cornea and
- a much thicker epithelium that covers the outside surface of the cornea

These cell layers work together to keep additional water from entering the cornea, which would result in swelling and opacity.

The epithelium also has a second function of providing a physical barrier against the entry of foreign materials. If the epithelium is injured, corneal opacity can result.

However minor opacities can often be reversed because the epithelium can recover itself either by movement of surrounding cells to cover the

wound or by the actual replacement of damaged tissue through new cell division. In contrast, the endothelium is generally not capable of self-healing. Therefore, if these cells suffer cytotoxic damage there can be significant consequences, e.g., permanent blindness.

It is this relationship between the induction of cellular damage and resulting ocular irritation or other injury that is the basis for *in vitro* ocular irritation methods.

Another delicate tissue of the eye is the conjunctiva, the non keratinized squamous epithelium that lines the inner surfaces of the eyelids and much of the external surface of the ocular globe (it is continuous with the cornea). The conjunctiva is highly vascularized and may become quite inflamed after exposure to irritating materials. Mildly irritating chemicals or other products often cause conjunctivitis without any associated corneal damage.

A third important ocular tissue is the iris (the colored part of the eye), which by constricting or dilating, controls the amount of light that enters the eye and is eventually focused on the retina. The iris lies under the cornea within the aqueous humor. In some cases foreign materials penetrate completely through the cornea and interact with the iris. The iris may then become very inflamed and may lose its ability to react to light, seriously damaging the ability to see.

Observations of the degree of injury to each of these tissues in the animal model are incorporated as part of the scoring system of most common eye irritation protocols.

Generally the process of substance testing consists of several steps

- First - the maximum potential hazard of the ingredient or formulation to the ocular tissue is determined.
- Second - the actual use of the product is considered, estimating the probability that it may inadvertently enter the eye.
- Third - a final safety assessment takes into account benefits, risks, and the impact of the instructions for use that generally accompany the product.

Although the entire process is important, it is the first stage of this process—generally termed hazard identification—and the development of improved *in vitro* systems to detect such hazards that are important.

Draize test

In Draize test, chemicals, mixtures, and formulations are introduced directly into the conjunctival sac of the rabbit eye. The other eye serving as the negative control, and the response of the animals is monitored using a standardized scoring system for injury to the cornea, conjunctiva, and iris. Ocular responses are scored at 1, 24, 48, and 72 hours. The animals are observed until the full magnitude and reversibility of the ocular injury can be evaluated—for up to 21 days.

The topical application of chemicals can cause irritation and/or corneal damage in several ways, including:

- lysis of membranes (e.g. by surfactants, organic solvents);
- denaturation of proteins (e.g. by surfactants, organic solvents, alkalis and acids);
- saponification of lipids (e.g. by alkalis); and
- alkylation or other covalent interactions with macromolecules (e.g. by bleaches, peroxides)

Reversibility of the ocular injury is an important component in the classification of a substance as an eye irritant versus an eye corrosive.

Known since 1944 Draize Eye Test Rabbit Irritation uses a complex scoring system that reflects the degree of damage to the three major tissues of the eye. Also, the reversibility and the severity of the effects are evaluated.

The modified Draize Test method is Low Volume Eye Test (LVET) method, which uses one-tenth of the material normally applied to the rabbit eye. LVET is reported to be better predict the response of human eyes and to be less hazardous to the animal.

The retrospective validation study of the refinement/reduction Low Volume Eye Test (LVET) method for the use domain of household detergents and cleaning products as well as their main ingredient classes took place between 2006 and 2009.

After peer review, the LVET was not recommended for prospective use, i.e. to generate new data but it was recommended that existing LVET data of the limited use domain of household detergents and cleaning products as well as their main ingredient classes may be used for purposes of classification and labeling decisions. Moreover, it was recommended that existing LVET data of this limited use domain may be used as supplementary data for future validation studies. No additional testing should be however performed to further develop or validate the LVET test (ESAC, 2009).

Why not rabbit

Mentioned above Draize Eye Test Rabbit Irritation uses a complex scoring system that reflects the degree of damage to the three major tissues of the eye.

Aside from the obvious ethical reasons, requiring the discontinuation of cosmetics testing on animals, the quality and accuracy of the results obtained by this method is also questionable.

Historically, the albino rabbit has been the animal of choice for testing potential eye irritants, because of size of the eyes, which make it easy to observe damage and size of conjunctival sac (accentuated by loose lids) that easily accepts test material.

However, because of several striking differences, the rabbit is far from the perfect model for humans.

The anatomy and biochemistry of the rabbit eye are not equivalent to those of the human eye.

Here are the differences:

- third eyelid, which moves laterally across the eye, likely causing removal of many test material, what differ from humans.
- conjunctival sac – much larger than in humans, what means more material can be placed in the rabbit's eye than would be likely to ever get into human eye during an accidental exposure. [100 ml of liquid or 100 mg of a solid]
- cornea – rabbit cornea is much thinner than humans
- production of tears - the rabbit produces fewer tears
- blink frequency
- ocular surface area

For these and other reasons the rabbit is generally considered an overly sensitive model for humans, what may be considered a positive aspect, because it adds a safety margin to the risk management, however it presents the problem of inappropriate hazard assessment and suggests that a more predictive model would be beneficial.

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