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Alternative methods of eye irritation testing in industrial toxicology

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Abstract

The work is devoted to the study of applicability and sensitivity of various biological models that are used for testing eye corrosion and irritability. For this purpose, three different *in vitro* methods have been selected that are used to reveal the potential of substances to cause eye damage or irritation. *In vitro* methods have some limitations compared to the *in vivo* classical model. A suitable combination of these methods can usually replace standard *in vivo* testing in rabbits. The various combinations of alternative methods were verified experimentally, and the results obtained were compared with the conclusions of an *in vivo* test. Historical test results in rabbits were used for this work and no test on laboratory animals was performed.

Abstrakt

Práce je věnována studiu citlivosti a využitelnosti různých biologických modelů, které jsou používány pro testování oční leptavosti a dráždivosti. Pro tento účel byly vybrány tří různé *in vitro* metody, které se používají pro odhalení potenciálu látek způsobit poškození nebo podráždění oka. *In vitro* metody mají v porovnání s *in vivo* klasickým modelem řadu omezení. Vhodnou kombinací těchto metod lze většinou nahradit standardní *in vivo* testování na králících. Experimentálně bylo provedeno ověření různých kombinací alternativních metod a porovnány dosažené výsledky se závěry *in vivo* testů. Pro tuto práci byly využity historické výsledky zkoušek na králících a nebyl proveden žádný reálný test na laboratorních zvířatech.

Keywords

Eye irritation, BCOP, HET-CAM, EpiOcular, Dreize test, alternative tests, biological models.

Klíčová slova

Oční dráždivost, BCOP, HET-CAM, EpiOcular, Dreize test, alternativní testy, biologické modely.

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Introduction

Contact of the chemical with the human eye can cause negative reactions, which are manifested by various intensities of irritation, inflammation, corneal damage, impaired vision and blindness. Evaluation of ocular irritancy is considered to be a key step in the safety assessment of a wide range of industrial chemicals and consumer products. The in-vivo Draize eye irritation test[1] and its numerous modifications[2], which were used for this purpose for many decades, gradually cease to meet the modern requirements for toxicological testing. Ethical[3] and legal[4] standards as well as methodological reasons have led to the search for more suitable alternatives. A number of techniques that use cellular, tissue or organ systems have been scientifically developed for eye irritation testing.

This work is focused on the use of alternative models as a replacement for in vivo testing. The theoretical part first describes the biological models used, their practical applicability, sensitivity, advantages and disadvantages.

HET-CAM, BCOP and RhCE methods using different biological models were selected for experimental work. The sensitivity of these tests was verified by comparison with the results of in vivo tests. Special attention was paid to the HET-CAM method, which is the only one that detects the effect of test substances on the conjunctiva. Various chemicals and mixtures with experimentally demonstrated in vivo effects on the eye were selected. Substances for which different degrees of conjunctival irritation were recorded were specifically targeted. For the purposes of this experiment, no in vivo test was performed and data from previous laboratory animal studies were used to compare the in vitro tests performed with the in vivo results.

Part of the experimental work was laboratory verification of the proposed modification of the BCOP test for colored and highly viscous substances. The OECD method is not recommended for this type of substance. By modifying the methodology and its subsequent validation, the applicability of this method to a wider range of substances was extended.

The obtained results of alternative tests were compared with in vivo experiments. A key factor was the comparison of the sensitivity of the biological model, but also the correlation with the observed response of individual tissues in the in vivo test. Another aim of the work was to propose a suitable combination of alternative tests, which would correspond as accurately as possible to the original in vivo test in rabbits.

1 Theory

1.1 In vivo eye irritation test

Draize eye irritation test in rabbits was accepted by the Organization for Economic Cooperation and Development (OECD) as Guideline Test No.405[2]. The method was used for testing eye irritation for all types of chemicals. The test substance is applied to the conjunctival sac of one eye (the other eye is a control for evaluating the ocular response). The degrees of conjunctival, corneal and iris damage are evaluated at various time intervals and then the total irritation score is calculated[5]. The advantage of this test over all published alternative methods is the complexity and systemic response of a living organism and the ability to evaluate the reversibility of changes in the cornea, iris and conjunctiva. The disadvantages of this in vivo test are, in addition to ethical reasons, the subjectivity of the evaluation, the anatomical difference of the rabbit eye, insufficient knowledge of the mechanism of action and high variability of results[16,17]. In alternative assays, the originally claimed economic advantage appears to be questionable because some of the in vitro biological models used (e.g., commercially produced 3D tissue models) are relatively expensive.

1.1.1 Alternative biological models

Alternative methods in the eye corrosion and irritation testing have been developed for a relatively long time and use various testing systems. They are most often divided into cellular, organotypic or tissue models.

Cell lines – 2D models
The simplest type of biological models for testing eye irritation are selected cell lines simulating the epithelium of the top layer of the cornea[8]. The methods are evaluated by the classical principle of cytotoxicity. Damage of cell membrane integrity as a result of contact with a chemical is monitored by Neutral Red Release Test and Neutral Red Uptake Test[9,10,11]. Another possibility is monitoring the disruption of binding between cell culture cells. This effect is detected by measuring the permeation of fluorescein through the cell layer[10,12,13]. So far, the only OECD recommended method for this type of biological model is the Fluorescein Leakage Test for the identification of corrosive and highly irritating substances (OECD TG. 460) from 2017[14]. The disadvantage of these models is limited applicability for some chemicals (volatile, insoluble, unstable in aqueous solution)[15,16]. It is also difficult to extrapolate the results to an animal model. The advantage is the test speed[17], the low price and, of course, the ethical aspect.

Three-dimensional tissue models
Tissue 3D models are a more complex type of alternative biological models for testing eye irritation. Several cosmetic companies have specialized in the development of a corneal surface model[18]. Epithelial 3D models are very fragile, which requires careful handling to damage the structure[19]. Another limitation is the modeling of only the epithelial layer, so they cannot be used to determine the possible effects of substances penetrating the stroma and endothelium. Irreversibility cannot be evaluated in these models[20,21]. Tests based on cell models lack the simulation of hormonal,

immune and nervous influences. Their advantage is the simplicity and good reproducibility of the results.

Isolated eyes

Ex vivo methods use isolated organs that retain some of the biological functions and properties of living organisms for up to several hours after killing the animal[22]. For eye irritation testing, whole rabbit and chicken eyes or isolated bovine and porcine corneas are used as alternative biological models for the cornea. The models are placed in specialized holders that allow them to retain their biological properties throughout the test. The principle of these tests is to monitor changes in the opacity and permeability of the cornea after application of the test substance. These are models for the cornea only without the possibility of monitoring the reversibility of the lesions and the systemic effect after ocular exposure[5]. Methods using whole chicken eye (ICE) and isolated bovine cornea (BCOP) have been included among the approved OECD test procedures (OECD TG 438 and 437)[22,23]. These methods are suitable for testing chemicals regardless of their solubility. Identification of mildly irritating substances is more difficult. The use of porcine cornea does not require prevention of encephalopathic disease compared to bovine cornea[24]. They are anatomically more similar to the human cornea and are used in ophthalmological research[25].

Fertilized bird eggs

The only scientifically validated model for the conjunctiva is the chorioallantoic membrane (CAM) of fertilized bird eggs. At a certain stage in the development of the embryo, the CAM resembles the vascular conjunctiva of the mammal[26]. The degree of damage is determined based on the rate of onset of the observed vascular lesions[27]. This test is one of the tests recommended by ECVAM, but has not yet been included among the OECD methods.

For the HET-CAM test, most comparative studies have shown a significant corelation between this alternative and the Draize test, especially for mildly irritating and non-irritating chemicals[28,29,30].

An interesting modification is the CAMVA test (chorioallantoic membrane vascular assay), which also uses the chorioallantoic membrane as a biological model, but in eggs 14 days after fertilization[31,32]. The use of the chorioallantoic membrane (CAM) is much broader than eye irritation testing. The highly vascularized membrane is used in medicine, bioengineering[33] and other areas of research.

1.2 Testing strategies for the use of alternative models

All alternative biological models focus on one specific type of damage. Alone they are not able to capture the whole spectrum of damage which is in contact with the human eye irritant or corrosive substances realistically occur. Currently, the development and modification of alternative methods aimed to create a corresponding set (battery) prediction models, covering the widest range of effects[34,35]. Current chemical legislation (REACH)[36] calls for consideration to be given to the need to test the hazards of chemicals using in vivo tests. Preferably, in vitro and ex vivo methods must be used if these methods are validated and required by Commission Regulation (EC) No 440/2008[37].

2 OBJECTIVES OF THE DISSERTATION

The aim of the work was to verify the sensitivity of three different alternative methods using different biological models in their use as a replacement for the in vivo method for testing eye irritation. The selection of methods included two alternative tests using corneal models and one test based on the human conjunctiva model. Experiments with alternative methods were performed in the SLP Testing Facility of the Research Institute of Organic Synthesis, a.s. v Rybitví, where all methodologies are standardly introduced into the certified system of Good Laboratory Practice. Available detailed in vivo test data were used to evaluate the results of alternative methods. The experimental phase can be divided into two specific objectives:

- Modify the existing BCOP method for testing coloured and viscous substances. For this type of substance, design and validate an extension of the rinsing portion of the test that reduces the risk of false-positive results due to the test substance adhering to or staining the cornea.
- To verify the sensitivity of three different alternative eye irritation tests and to suggest a suitable combination that would be as comparable as possible to the in vivo rabbit test.

3 EXPERIMENTAL PART

3.1 Eye irritation testing methodologies

3.1.1 In vivo eye corrosion/irritation test

The test model was albino rabbits regardless of sex. The chemical was applied in the prescribed dose to the conjunctival sac of one eye of the experimental animal. The other eye of this animal served as a chemical-free control. At intervals of 1, 24, 48 and 72 hours after exposure, lesions on individual parts of the eye (conjunctiva, cornea, iris) and other signs of irritation (tearing, swelling, systemic toxicity) were observed and recorded. Initially, the application was performed on only one animal. A maximum of three rabbits were used in each test. The ocular response was monitored for up to 21 days. The reversibility of the lesions was an important indicator. Numerical ratings were assigned to the observed changes with respect to the degree of response or damage. The scoring system prescribed by the OECD and EU methodologies was used for the evaluation. The irritancy index for each animal was obtained by summing all assigned values in the individual monitored time intervals. Based on the resulting average irritancy index, the irritant potential of the test substance was evaluated. For the purposes of this work, a classification system valid at the time of in vivo tests was used.

3.1.2 Test BCOP (Bovine Corneal Opacity and Permeability)

The biological model was an isolated bovine cornea. The collected eyes were transported in HBSS solution with the addition of antibiotics and on dry ice. The test was always performed on the day of eye collection. The defect-free corneas were cut with a 2-3 mm white margin and fixed in special holders that consisted of an anterior (epithelial) and posterior (endothelial) chambers. Both chambers were filled with EMEM solution and incubated in a water bath (32 ± 1 °C). After preincubation, the medium was changed in both chambers and the basal opacity of each cornea was measured. Corneas with an opacity value > 7 were excluded from the test. Selected corneas were sequentially divided into groups: negative control, positive control, and test substances.

The test substance (750 µl) was applied to the epithelial side of the cornea. The exposure time was 10 minutes for liquids and semi-solids or 4 hours for solids. After exposure, the cornea was washed at least 3 times with EMEM medium with and without phenol red. Opacity was measured with an opacitometer as a dimensionless number. The measurement was always performed at the beginning of the experiment and then at the end of the experiment. After measuring the opacity, EMEM medium was thoroughly aspirated from the anterior chamber and 1 ml of sodium fluorescein solution (5 mg / ml) was added, i.e. to the epithelial side of the cornea, while the posterior chamber was still filled with fresh EMEM medium. The holder was incubated in a horizontal position for 90 ± 5 minutes at 32 ± 1 °C.

After incubation, the solution was removed from the posterior chamber and the absorbance was measured using a UV / VIS spectrophotometer (OD490). A 1 cm thick cuvette was used in the tests, so the measured absorbance value was equal to

the optical density value. This value quantified the permeability of the cornea to fluorescein. Thus, an individual permeability value was obtained for each cornea.

Evaluation

- The opacity values were first adjusted for background turbidity, i.e., the opacity value obtained before application was subtracted from the opacity value after application for each cornea. This gave an individual adjusted opacity O ($_{ind}$).
- Subsequently, the average opacity was calculated for each corneal group (NK / PK / TL) (Ø O).
- Then, the mean values for the test and positive substance were corrected by subtracting the mean value for the negative control **O (cor)** $_{PK/TL}$
- For permeability, average values for tissues affected by test substance or positive control substance were calculated from individual values: Ø **P** $_{TL/PK}$
- The average permeability was then adjusted by subtracting the average permeability of the negative control (solvent): **P (cor)** $_{PK/TL}$

$$IVIS = \textbf{Opacity (cor)}_{PK/TL} + (15x\ \textbf{Permeability (cor)}_{PK/TL})$$

The final results were expressed as an in vitro irritancy score (IVIS), which was used to assign the irritant potential of the substance. The standard GHS classification was used for this classification. Substances with an irritation score value less than or equal to 3 are considered non-irritating. Substances with an IVIS value higher than 55 must be classified as seriously damaging to the eye (Category 1). Substances with an IVIS parameter between 3 and 55 cannot be classified based on the BCOP test and further in vitro or in vivo tests are required.

Modification of rinsing procedure

According to the OECD test guideline[23], critical factors of the BCOP test are ensuring that the test substance adequately covers the epithelial surface and that it is adequately removed during the rinsing steps[21]. Highly viscous pastes and oily substances often exhibit strong tendency to stick on the cornea surface and it is difficult to wash them away using the standard procedure. Prolonged contact of highly lipophilic compounds with corneal tissue may subsequently lead to the enhanced penetration[37]. Coloured substances in turn can cause unrecoverable staining of cornea. All of these phenomena can give rise to false positive results, which we have also noticed several times during the testing of chemicals for the purposes of REACH. To eliminate this limitation, a modification of the washing process was validated at the beginning of the experimental work, which included not only gentle mechanical removal of the applied substance, but also the inclusion of the lipophilic substance in the washing cycle. The process was verified on a set of 20 substances. This step was validated and subsequently included in the internal methodology in the SLP system at the VUOS workplace[38]. It is currently used as standard for commercial eye irritation testing.

3.1.3 RhCE test (Reconstructed human Cornea-like Epithelium)

For the experimental phase, an EpiOcular™ tissue model from human epidermal keratinocytes, which form a stratified squamous epithelium simulating the corneal epithelium, was used. The experiments were performed according to the Standard Work Procedure, which was verified under laboratory conditions.

The whole course of the experiment took place under sterile conditions. All test substances were applied undiluted (50 µl or 50 mg). The exposure time was 30 minutes for liquids and 90 minutes for solids. After washing, postincubation was continued for another 2 hours for liquid test samples or 18 hours for solid test samples. After this postincubation, the tissues were dried and transferred to MTT solution, where they were stained. To determine the intensity of the staining, the dye had to be extracted from the tissues with isopropanol for 2-3 hours. Isopropanol was decanted from each tissue and 2x200 µl was collected from the extract into 2 wells in a 96-well plate. The absorbance of the extracts was measured at 570 nm on an Epoch spectrophotometer.

To assess the validity of the test, a positive control (methyl acetate) recommended by the methodology was always included in the group of test substances. The results of all negative and positive controls met the prescribed criteria.

Evaluation

- The average blank value was subtracted from each OD value in the experiment (blank-corrected values).
- The average value of the corresponding tissues was calculated for each control and each test substance (average value for test substance / control).
- The corrected OD value of the negative control corresponded to 100% viability.
- The percentage viability of the corresponding tissues for controls and test substance relative to the negative control (100%) was calculated.
- The test substance was classified according to GHS

3.1.4 Zkouška HET-CAM (Hen´s Egg Test – Chorioallantoic Membrane)

HET-CAM is an alternative "in vitro" method that uses the chorioallantoic membrane of hens' eggs. The methodology used was based on the ICCVAM protocol[39]. After testing, demonstrably fertilized eggs from Leghorn hens weighing 50 to 60 g were used. After transport to the laboratory, the eggs were incubated under the prescribed temperature and humidity conditions. On the eighth day of incubation, the boundary of the air bubble was marked on the shell. On the ninth day of incubation, the eggs were removed and divided into groups: negative control, positive control and test substances (3 eggs per group). All eggs had a hole in the shell so as not to injure the inner paper membrane. The membrane was then soaked in saline and the eggs returned to the hatchery. After a 30 minute incubation in the hatchery, the saline was removed and the paper membrane was removed with tweezers. This revealed a negative and positive control for the chorioallantoic membrane to which the test substance was subsequently dosed.

Liquids and pastes were applied in an amount of 0.3 ml using a pipette. The crushed solids were dosed in a volume of 0.3 ml or 0.3 g. In each case, the amount of substance was sufficient to cover 50% of the membrane area. After application of the test substance, the membrane surface was monitored using a Leica stereomicroscope with a camera. Reliability check (test validity) was performed by simultaneous testing of positive (1% NaOH) and negative control (0.9% NaCl).

Evaluation
A stereoscopic microscope at 80x magnification was used for the evaluation. The presence and rate of onset of the following changes were monitored on the membrane vessels for a specified period of 300 s:

1) hemorrhage
2) vascular lesions
3) coagulation (denaturation of vascular proteins)

The observation time of the chorioallantoic membrane of the hen's egg did not exceed 300 s. Numerical values were assigned according to the rate of lesion onset. The result was the sum (irritation score) indicating the irritation potential of the test substance on a scale with a maximum value of 21.

3.2 Test substances

3.2.1 Modification of the BCOP method - optimization of the test substance rinsing procedure from the corneal surface

A group of 20 viscous substances with different colours was selected. The key parameters were density and consistency. For chemicals and pharmaceuticals, the irritant potential of an in vivo test was known. For cosmetic products, this information was logically not available, but the advantage of these samples was the pasty consistency and the colorant contained. It is known from laboratory practice that these substances cannot be tested by the standard BCOP method.

3.2.2 Verification of sensitivity of in vitro test methods

Various chemicals and mixtures with known in vivo effects on the eye were selected for laboratory experiments. A key indicator for the selection of substances was the availability of detailed results of the rabbit eye irritation test. The group included substances that irritate or damage various parts of the eye as well as non-irritants. Data on substances registered under REACH[5], which are available on the ECHA website, were used to evaluate the in vivo response. Furthermore, the results of studies for which primary records were available were used. An overview of the substances used and the results of the in vivo tests are given in Table 1.

Table 1: Overview of substances used to verify the sensitivity of in vitro test methods

No.	appearance	result in vivo	Cornea reaction	Conjuctiva reaction
1	solid/white-grey	non-irritating	--	--
2	solid/ white	irritating	medium	medium
3	solid/ white	non-irritating	--	mild-medium
4	liquid/ yellow	extremely irritating	significant	significant
5	solid / violet	mildly irritating	mild	mild
6	solid/ white	non-irritating	--	mild
7	solid/ white	non-irritating	--	mild
8	solid/ white	irritating	--	medium
9	solid/ white	non-irritating	mild	mild
10	solid/ green	severely irritating	significant	significant
11	solid/ white	severely irritating	significant	significant
12	solid/ brown-grey	non-irritating	mild	medium
13	solid/ black	non-irritating	--	mild
14	solid/ yellowish	irritating	--	medium
15	solid/ white	mildly irritating	mild-medium	medium
16	liquid/ dark brown	non-irritating	--	mild
17	liquid/colorless	non-irritating	--	mild
18	liquid/colorless	non-irritating	--	mild
19	liquid/colorless	irritating	mild	significant
20	solid /green-black	mildly irritating	mild	medium
21	solid/ white	irritating	mild	mild-medium
22	solid/ white	irritating	medium	medium-significant
23	solid/ white	non-irritating	--	mild
24	liquid/ yellow	non-irritating	--	mild
25	solid/white-grey	non-irritating	--	mild-medium
26	solid/brown	mildly irritating	--	mild-medium
27	solid/white-grey	irritating	mild-medium	medium
28	solid/ green-yellow	non-irritating	--	mild
29	solid/ green-yellow	mildly irritating	--	mild-medium
30	liquid/colorless	mildly irritating	--	mild-medium

4 Results and discussion

4.1 Modification of the BCOP method - optimization of the test substance rinsing procedure from the corneal surface

Highly viscous and coloured 20 substances selected for modification of the BCOP test were first tested by the BCOP test, according to the OECD test procedure, i.e. with a standard triple rinse with EMEM medium. The next step in verifying the proposed modification was to use the BCOP test with a modified rinse. An olive oil rinse was inserted between the standard EMEM medium. The aim was to increase the efficiency of the washing process for substances soluble in non-polar solvents, especially coloured substances. In the third step of the experiment, the same group of substances was tested by another variant of the BCOP test, in which mechanical removal of residues of the substance from the corneal surface was performed before the rinsing part using a cotton swab. This step was again followed by an extended rinsing process with a combination of EMEM medium and olive oil. This modification has been proposed for highly viscous substances.

The averages of the measured opacity and permeability values and the corresponding calculated IVIS scores are summarized in Tables 2 and 3. Each experiment (standard BCOP rinsing, non-polar rinsing modification and mechanical corneal removal modification) was performed on a group of five corneas that were independently evaluated. Thus, a total of 15 corneas were used for each substance. The results of all three variants were compared not only with each other, but also with the expected irritant potential of the tested substances. As part of the verification, the effect of both steps on intact corneas was verified, thus eliminating the possibility of a false positive effect of both modifications.

When performing a standard rinse (3x EMEM), residues of test substances on the surface of the corneas were observed in 11 samples (55%). In two cases, the cornea remained stained with the sample. The use of a non-polar solvent rinse has increased the efficiency of the washing process. Residues of test substances were observed in 7 samples (35%) and corneal staining was recorded in only one sample. When mechanical removal of the substance from the corneal surface was included, residues of the applied sample were detected only in sample No. 1 (5%).

***Table 2**:* Modification of the BCOP test - Average values of opacity and permeability

No.	rinsing EMEM			rinsing EMEM+oil			mechanical removal + rinsing EMEM +oil		
	OP	PER	residues	OP	PER	residues	OP	PER	residues
1	55.2	0.039	+ B	66.2	0.057	+ B	36.6	0.052	+
2	53.4	0.012	+ B	5.4	0.009	N	6	0.01	N
3	101	0.021	+	82.4	0.012	+	13	0.011	N
4	38	0.017	+	9.8	0.026	N	2.2	0.014	N
5	9,6	0.067	N	37	0.012	N	10,4	0.023	N
6	3	0.023	N	10.6	0.023	N	12	0.054	N
7	209.4	0.089	+	214	0.071	+	79.4	0.023	N
8	204.6	0.013	+	229.2	.,02	+	71.2	0.054	N
9	18.4	0.185	N	43.2	0.039	N	29.6	0.032	N
10	11.6	0.084	N	23.6	0.029	N	6.4	0.013	N
11	9.8	0.043	N	6	0.037	N	7.6	0.065	N
12	202.4	0.016	+	20.4	0.064	N	5	0.041	N
13	8.8	0.047	N	15.4	0.032	N	10.2	0.014	N
14	141.2	0.026	+	8.4	0.014	N	9.6	0.032	N
15	168	0.026	+	160.8	0.012	+	21	0.019	N
16	15	0.013	N	50.8	0.027	N	11	0.054	N
17	227.2	0.023	+	232.4	0.015	+	23.2	0.009	N
18	16	0.018	N	15.4	0.022	N	7	0.009	N
19	113.4	0.026	+	115.8	0.093	+	80.4	0.045	N
20	19.4	0.776	N	19.4	0.850	N	13.4	0.611	N

Note: OP - average opacity; PER - average permeability; + - presence of test substance on the cornea after the washing phase; B - persistent corneal staining after the washing phase; N - without residues of the substance on the cornea

When performing the standard BCOP test, the irritant potential (IVIS value greater than 55) was measured in nine samples, ie 45% of the substances. In all these samples, the presence of the test substance on the surface of the corneas was recorded

even after triple washing with EMEM solution. The use of the first modification led to a significant reduction in the calculated IVIS values for two samples (Nos. 12 and 14). The substance was completely removed from the corneal surface. In the other seven samples (Nos. 1, 3, 7, 8, 15, 17 and 19) where the IVIS value was higher than 55, there was no significant reduction in the value of the irritation score. The other four samples had a reduction in the calculated IVIS (Nos. 2, 4, 11, 18). The most significant decrease in irritancy index was observed for samples where modification caused removal of the test substance from the cornea. In contrast, the use of olive oil led to an increase in the IVIS value in six samples (Nos. 5, 6, 9, 10, 13 and 16), although it did not increase the limit value above 55 in any of them. significant changes.

The second modification of the test was based on a combination of mechanical removal of test substance residues from the corneal surface and subsequent rinsing with EMEM medium solution and olive oil. Of the seven samples for which the first modification did not reduce the IVIS score (Nos. 1, 3, 7, 8, 15, 17, 19), the inclusion of mechanical removal in the wash phase caused IVIS to fall below the limit in four of them (Nos. 1, 3, 15 and 17). In all but No. 1, the samples were completely removed from the corneal surface. For samples 7, 8 and 19, the IVIS value remained higher than 55, but the inclusion of the second modification in the test caused a significant reduction in this value. In contrast, an increase in IVIS in four samples (Nos. 5, 6, 9, 13). For all these substances, the newly obtained score value was well below 55.

***Tabulka 3**:* Modification of the rinsing phase of the BCOP test - Average IVIS values

No.	rinsing EMEM	rinsing EMEM + oil	Mechanical removal + rinsing EMEM +oil
1	**55.78***	**67.06***	37.39
2	53.58	5.4	6.16
3	**101.31***	**82.59***	13.17
4	38.26	10.2	2.41
5	10.6	37.17	10.74
6	3.35	10.94	12.81
7	**210.73***	**215.07***	**80.08***
8	**204.79***	**229.51***	**72.16***
9	21.18	43.79	30.08
10	12.86	24.04	6.6
11	10.45	6.56	8.58
12	**202.64***	21.35	5.61
13	9.51	15.87	10.42
14	**141.6***	8.61	10.08
15	**168.38***	**160.99***	21.29
16	15.19	51.21	11.82
17	**227.55***	**232.63***	23.34
18	16.28	15.72	7.14
19	**113.79***	**117.2***	**81.07***
20	31.04	31.15	22.57

*Note * The substance can be classified according to GHS*

For the first experimental part of the work, two changes were proposed to the washing process of the BCOP test, which was standardly performed according to the

OECD test method. The aim of the modifications was to increase the efficiency of removing colored and viscous test substances from the corneal surface. Residues of these substances increased the measured opacity values and thus caused false positive results[23]. The first modification based on the use of olive oil as a non-polar washing medium caused a significant decrease in IVIS values in one fifth of the samples (Nos. 2, 4, 12 and 14). This effect was related to a reduction in the presence of test substance residues on the corneal surface and a related increase in light transmission. The measured opacity values were significantly lower than when using the methodology prescribed by the methodology. The opposite effect was observed in two samples (Nos. 5 and 16) when the use of olive oil to remove the test substance from the surface caused an increase in the IVIS value. In these cases, there was only an increase in the measured opacity value, but the permeability values were not significantly affected. Dissolution of non-polar substances in olive oil could cause them to penetrate the upper layers of the cornea and thus reduce light transmission. A possible explanation could be the effect of olive oil alone, but this has not been confirmed in chemical-free control tests. The use of oil in the rinsing step did not increase the IVIS value above the limit of 55, so there was no false positive response.

Increased permeability was observed in two samples (Nos. 12 and 19) using the first modification. For sample No. 12, this effect was balanced by a decrease in opacity values and thus an overall decrease in the IVIS score. In contrast, sample No. 19 showed a slight increase in the irritancy score. The effect of both modifications on the permeability values was not observed for the other samples.

Mechanical removal of test substance residues from the corneal surface before an extended sequence of rinsing steps led to a significant increase in the removal efficiency of test substances whose residues caused corneal opacity and thus an increase in the resulting IVIS score in four samples (Nos. 1, 3, 15 and 17). Also in these cases, no significant change in corneal permeability was observed.

To verify the effect of the modifications themselves on the compactness of the corneal epithelium, tests without chemicals and tests with three positive controls were performed. In all cases, the application of mechanical contact and the inclusion of olive oil in the washing process alone did not have a significant effect on the measured values of opacity and permeability. Histopathological examination of exposed corneas also showed that the modifications did not cause disruption of the corneal epithelium.

4.2 Verification of sensitivity of in vitro test methods

4.2.1 HET-CAM test

A group of 30 samples (Table 1), which were selected to verify the sensitivity of in vitro methods for testing eye irritation, were tested by the HET-CAM test. No chorioallantoic membrane response was observed in Samples 1, 7, 12, and 16. A very slow onset of vascular dilatation (after 135 seconds) was observed for substance No. 6 in only one of the three eggs tested. All of these substances were classified as non-irritating to the eye.

Vascular changes were the fastest and always the first to be observed (in the HET-CAM test they are dilatations of blood vessels or their vasoconstriction). These lesions are considered to be the mildest and their score for calculating the irritation

score is the lowest. In several samples (Nos. 8, 13, 17, 18, 20, 21, 24, 25 and 28) only this reaction was observed, when there was a gradual dilation of blood vessels of varying speed, without the onset of hemorrhage and coagulation. The calculated IS values were relatively low (1.3 - 5).

The most frequently observed combination of two lesions was dilatation of blood vessels, gradually progressing to the integrity of the vessel and bleeding. The time interval between the onset of both lesions was about 20 seconds. These changes were observed for one third of the tested substances (Nos. 2, 3, 5, 9, 10, 15, 23, 26, 29 and 30). Their final irritation score was 6.7 - 11.3.

Samples Nos. 4, 11, 14, 19, 22 and 27 not only showed vascular changes and hemorrhage, but gradually showed coagulation of proteins around the vessels. These changes are considered the most serious. The resulting irritation score for these substances was higher than for the other samples and ranged from 11 to 21.

***Table 4*:** HET-CAM test results

No.	IS	classification	No.	IS	classification
1	0	non-irritating	16	0	non-irritating
2	9.3	corrosive / severely irritating	17	2	mildly irritating
3	10.7	corrosive / severely irritating	18	5	moderately irritating
4	21	corrosive / severely irritating	19	16	corrosive / severely irritating
5	10.7	corrosive / severely irritating	20	4.3	mildly irritating
6	0.3	non-irritating	21	5	moderately irritating
7	0	non-irritating	22	17.7	corrosive / severely irritating
8	5	moderately irritating	23	8.3	moderately irritating
9	10.7	corrosive / severely irritating	24	3.7	mildly irritating
10	10	corrosive / severely irritating	25	5	moderately irritating
11	19	corrosive / severely irritating	26	10.7	corrosive / severely irritating
12	0	non-irritating	27	11	corrosive / severely irritating
13	1.3	mildly irritating	28	2	mildly irritating
14	11.3	corrosive / severely irritating	29	11.3	corrosive / severely irritating
15	6.7	moderately irritating	30	10.7	corrosive / severely irritating

One of the aims of this work was to compare the results of individual alternative methods with the results of in vivo tests. For substances that were classified as very severe irritant (No. 4) and very irritating (Nos. 10 and 11) based on the classical test in rabbits, there was 100% agreement in the results of the classical test in rabbits and the HET-CAM method because The calculated irritation score for the alternative test could all be classified as corrosive / severely irritating.

For samples with an in vivo classification of "eye irritation", the result of this alternative test was more stringent. For samples 2, 14, 19, 22 and 27, the calculated irritancy scores were higher than 11, so they were also classified as corrosive / severely irritant. Thus, the HET-CAM test was more sensitive for these samples. A more accurate in vitro response was observed for two irritants (Nos. 8 and 21). The IS was the same for both substances - 5 and the substances were classified as moderately irritating based on the results of the alternative method. Even in this group of substances, the result can be considered relatively identical when compared to in vivo classification

A large difference and variability in the results was found in the group of substances that were classified as mildly irritating according to the results of in vivo tests (Nos. 5, 15, 20, 26, 29 and 30). The agreement was only in the sample No. 20, when according to the value of IS (4.3) the substance was also marked as mildly irritating. For other samples, the HET-CAM method was significantly more sensitive. Sample No. 15 with an IS value of 6.7 was classified as moderately irritating, although the irritation score value was only slightly above the limit for mildly irritating substances. The other samples had an IS value of 10.7 or 11.3 and were classified as corrosive / severely irritant. For these substances, it is interesting to compare the lesions observed in in vivo tests. All samples showed more pronounced conjunctival damage compared to the corneas (No. 15) or only the conjunctiva (No. 26, 29 and 30) was damaged, ie the part of the eye for which the chorioallantoic membrane is a model. This model should be more sensitive to these changes. Only in sample No. 5, slight changes in both the conjunctiva and the cornea were observed in rabbits. If we include detailed results of clinical observation in the overall assessment of the sensitivity of the HET-CAM method for a group of mildly irritating substances in in vivo tests, one of the reasons for the increased sensitivity of the alternative method can be considered a higher prediction for substances that irritate the conjunctiva.

The last evaluated group are substances which were classified as non-irritating to the eye on the basis of the classical test (Nos. 1, 3, 6, 7, 9, 12, 13, 16, 17, 18, 23, 24, 25 and 28). For these substances, the agreement was 36% in the result. IS less than 1 was calculated for substances 1, 6, 7, 12 and 16. Based on the result of the HET-CAM test, four samples (Nos. 13, 17, 24 and 28) were classified as mildly irritating and three as irritant. samples (Nos. 18, 23 and 25). For all of these substances, changes in the conjunctiva were observed during in vivo exposure, but did not lead to classification by any degree of irritation. This result is consistent with the hypothesis of sensitivity of the chorioallanthione membrane model to conjunctival damages set forth above. For samples No. 3 and 9, the biggest difference was in the results. Both substances were non-irritating in in vivo tests, but would be classified as corrosive / severe irritant based on the HET-CAM method. In both cases, slight changes in the conjunctiva and in sample No. 9 on the cornea were also observed in rabbits.

4.2.2 BCOP test

The aim of the experiments was to compare the results of the BCOP method and an in vivo test in rabbits in which the test substance is administered undiluted to the animals. For this reason, the “open chamber” method was used to test all solid samples by BCOP, where the solid is applied undiluted to the epithelial surface of

the cornea through the anterior opening of the holder chamber. The duration of exposure was 4 hours. Most solid samples (Nos. 1-4, 6-9, 11, 12, 14, 15, 21-23, 25-29) were followed by washing with EMEM medium as for liquids. During the exposure period, samples No. 10, 13 and 20 adhered to the surface of the cornea or slightly stained it. For this reason, a modification of the washing procedure was used to remove the substance from the cornea, which was designed and verified in the first part of the experimental work. The respective negative (0.9% NaCl) and positive control substances (100% dimethylformamide for liquids and 20% imidazole for solids) were always included in each group of test substances.

Opacity was measured for all samples tested, negative controls and positive controls with the MC2 opacitometer. The highest opacity values (higher than 50) were measured for samples 4, 5, 8, 10, 11 and 19. Conversely, for substances 3, 7, 16, 17 and 26, the opacity values were measured before and after exposure to the test substance. almost identical and did not exceed.

Membrane permeability was determined for all 30 samples in the same manner by applying 1 ml of sodium fluorescein solution to the epithelial side of the cornea. To measure absorbance, medium was removed from the posterior chamber after 90 minutes of exposure. The contents were analyzed with a Genesys UV / VIS spectrophotometer at 490 nm. Elevated permeability values indicating and permeation of flurescein through the cornea were detected in only a few samples (Nos. 4, 10, 11 and 14). These values indicate a violation of corneal tissue integrity.

The calculated irritation score values and the assigned classification of eye irritation potential are summarized in Table 5. A clearly negative result was found in six samples, where the IVIS value ranged from 0.33 to 2.69. Based on the GHS classification, these samples can be classified as non-irritating. In contrast, the lassification “severely irritating to corrosive to the eye” was assigned to all tested samples with an irritation score value higher than 55, which were samples No. 4, 10 and 11 with IVIS values of 134.06; 129.2 and 76.84. The highest opacity values were measured in these samples, so the test substance significantly reduced corneal transparency. The opacity of the corneas was visible after the end of the exposure and their washing. .

In none of the experiments did the corneas stain the test substances, nor were there any visible residues on the epithelial surface, which would indicate a false positive test result. When comparing the results of the bovine cornea test with the results of in vivo tests, 100% agreement was found for substances which are very irritating to the eye (No. 4) and severely irritating to the eye (Nos. 10 and 11). Only for these substances was the average IVIS value higher than 55 and the substances were classified as severely damaging to the eye to corrosive on the basis of the BCOP test.

***Table 5**:* BCOP test results

No.	IVIS	classification	No..	IVIS	classificatin
1	2.69	non-irritating	16	0.59	non-irritating
2	3.77	cannot be evaluated	17	0.67	non-irritating
3	1.33	non-irritating	18	2	non-irritating
4	134.06	corrosive / severely irritating	19	49	cannot be evaluated
5	33	cannot be evaluated	20	19.82	cannot be evaluated
6	2.36	non-irritating	21	12.69	cannot be evaluated
7	0.33	non-irritating	22	19.33	cannot be evaluated
8	42.94	cannot be evaluated	23	2.38	non-irritating
9	4.34	cannot be evaluated	24	2.7	non-irritating
10	129.2	corrosive / severely irritating	25	2.09	non-irritating
11	76.84	corrosive / severely irritating	26	11.7	non-irritating
12	0.33	non-irritating	27	32.38	cannot be evaluated
13	2.34	non-irritating	28	8.67	non-irritating
14	39.48	cannot be evaluated	29	11.09	cannot be evaluated
15	36.33	cannot be evaluated	30	41.48	cannot be evaluated

Almost 100% accuracy was also observed for in vivo non-irritants. Of the fourteen non-irritants, a negative result in the BCOP test was recorded in thirteen (Nos. 1, 3, 6, 7, 12, 13, 16-18, 23-25 and 28). For Sample No. 9, the IVIS value was only slightly above the limit for non-irritants.

The BCOP test is not sensitive enough for substances with a lower potential to cause eye irritation. For irritation score values above 3 and below 55, the result cannot be used to estimate the irritant potential of the test substance. Chemical legislation in these cases prescribes to continue testing using other alternative methods or the in vivo method in rabbits.

The experiment shows that the BCOP test is sensitive enough to detect non-irritating substances and substances that are more harmful to the eye. No false negative result was observed for these substances. It is not sensitive enough for other substances, as shown by the calculated IVIS values. The irritation score for in vivo mild eye irritants ranged from 11.09 to 41.48. For irritants, the variability of the irritation score was greater (3.77 - 49).

4.2.3 RhCE test

Chemicals (Table 1) were tested in two independent experiments in the EpiOcularTM model. An overnight tissue preincubation procedure was used for all samples. Liquids (50 µl) were pipetted directly onto the tissues. The solids (50 mg) were transferred with a spoon and carefully spread over the entire surface of the model. When applying the test substances, it was necessary to use a fixation mesh for sample No. 17, which ensured even coverage of the whole tissues. The duration of exposure was different for liquids and solid samples, as described in the methodology in Chapter 4.1.3. Testing of

some solids (Nos. 10, 12, 13, 20 and 26), which adhered to the surface of the model during exposure and could not be completely removed, proved to be problematic. After the post-incubation phase, MTT staining was performed for 3 hours followed by extraction with isopropanol. The absorbance of the extracts in a 96-well plate was measured with an Epoch spectrophotometer at 570 nm. The results were compared with the values of the respective negative control substance (sterile deionized water) and the average percentage of tissue viability after exposure to the test substances was calculated. The resulting values as well as the corresponding evaluation of the eye irritation potential are given in Table 6.

Only four samples (Nos. 1, 9, 16 and 17) had tissue viability higher than 60% of the control tissue. These substances do not need to be classified for any degree of eye irritation. The other 26 samples had tissue viability below this value and can be considered irritant based on the results of the RhCE test. For half of the samples the viability was less than 10% (Nos. 2-4, 7, 8, 11-14, 20, 24-28) and for the other substances it was in the range of 11.7 - 55.6.

Table 6: RhCE test results

No.	% viability	classification	No.	% viability	classification
1	91.1±11.67	non-irritating	16	69.8±3.04	non-irritating
2	0.9±0.99	irritating	17	90.5±4.6	non-irritating
3	1.6±0.21	irritating	18	33.3±4.03	irritating
4	0.6±0.21	irritating	19	11.7±0.85	irritating
5	55.6±4.24	irritating	20	0.9±0.28	irritating
6	53.7±1.77	irritating	21	31.8±1.48	irritating
7	2±0.64	irritating	22	14.6±1.06	irritating
8	6.2±2.83	irritating	23	32.1±2.62	irritating
9	89.5±6.51	non-irritating	24	0.8±0.49	irritating
10	30.2±7.42	irritating	25	2.1±0.64	irritating
11	0.8±0.57	irritating	26	0.6±0.71	irritating
12	1.1±0.57	irritating	27	2.2±0.71	irritating
13	1.5±0.57	irritating	28	8.2±0.78	irritating
14	2±0.92	irritating	29	32.2±4.38	irritating
15	43.4±2.47	irritating	30	22.6±0.07	irritating

The work confirmed that the method using the reconstructed human cornea is significantly more sensitive than other alternative methods and the original in vivo test. For non-irritants, agreement was recorded in only 29%. A false positive result was recorded for ten samples.

All in vivo irritants were positive in the RhCE test and would be classified as eye irritants based on GHS. Based on the determined values of tissue viability, it is currently not possible to assign any qualitative degree of irritation to the tested chemical substances or to classify the substances as corrosive to the eye.

4.3 Comparison of in vivo and in vitro test results

The results of eye irritation testing using alternative methods on a group of 30 substances confirm the expected difference in the sensitivity of selected in vitro models.

The HET-CAM test, which has a high predictive capacity for substances that are highly irritating to the eye and a relatively good predictive value for substances that are slightly irritating and non-irritating, appears to be the closest to the classical test in rabbits. However, this test has not yet been sufficiently validated and is therefore not one of the methods recommended by the OECD. The BCOP test, which is based on the use of an isolated bovine cornea as a biological model, is very suitable for the identification of highly irritating and non-irritating substances. For this reason, the OECD methodology focuses only on the identification of these substances. For substances with a mild and moderate potential to cause eye irritation, it is inaccurate and, according to the eye irritation testing strategy, the second step after the BCOP test is the RhCE test with a 3D corneal tissue model. The EpiOcular model was experimentally applied to a selected group of chemicals and proved to be extremely sensitive. According to the results of this method, 26 substances were classified as irritant and only 4 as non-irritating eye.

Comparing the sensitivity of individual alternative methods is problematic because their classification scales are very different. The HET-CAM test from the achieved IS distinguishes 4 degrees of irritation (Table 5) and allows the classification of substances according to the degree of eye irritation that the test substances can cause. The alternative BCOP test using bovine cornea classifies only substances that are highly irritating to corrosive to the eye and non-irritants. A large group of substances is included in the category "cannot be evaluated". Using this classification scale, it is difficult to compare the results of substances causing mild to moderate eye irritation under in vivo conditions. The RhCE test has a very strict classification, which divides substances only into non-irritants and irritants. Due to the high sensitivity of this test, substances classified as irritant include not only substances with different potential for eye irritation, but also some non-irritant substances in vivo. Extreme sensitivity was also confirmed in the experiments of this work. Inconsistencies in the depth of resolution of the degree of irritability make it difficult to compare the results of individual alternative methods. If we divide the results into only positive (irritability regardless of the degree) and negative (non-irritant), the sensitivity of biological models to the in vivo test in rabbits can be partially assessed. This distribution is indicated in Table 14 by the gray of the positive fields. For the HET-CAM test, the agreement is 70% and for the RhCE test it is 66.7%. By including the controversial results of the BCOP test ("cannot be evaluated") among the positive results, the agreement will increase from 46.7% to 93%.

If we use the BCOP test as the first step of the testing strategy, substances that are highly irritating and non-irritating will be identified. According to the REACH testing strategy, the use of a second in vitro method is recommended to test the eye irritation potential of other substances. To assess the sensitivity of the RhCE and HET-CAM methods for a group of 13 substances that cannot be evaluated based on the BCOP test (No. 2, 5, 8, 9, 14, 15, 19-22, 27, 29 and 30), a comparison of the results was performed. of this subgroup with rabbit test results. The agreement of the results of this subgroup with the in vivo result was 100% for RhCE and 92% for HET-CAM. In neither case was there a false negative classification of the irritant as a non-irritant. These results confirm that although both methods provide information on damage to another type of ocular tissue, their overall sensitivity is comparable[40]. For this reason, the RhCE test is also included in the strategy of gradual eye irritation testing. In contrast

to the HET-CAM method, it is a method validated and included among the OECD recommended methods.

***Table7**:* Summary of in vivo and in vitro test results

No.	in vivo	HET-CAM	BCOP	EpiOcular™
1	non-irritating	non-irritating	non-irritating	non-irritating
2	irritating	corrosive / severely irritating	cannot be evaluated	irritating
3	non-irritating	corrosive / severely irritating	non-irritating	irritating
4	extremely irritating	corrosive / severely irritating	corrosive / severely irritating	irritating
5	mildly irritating	corrosive / severely irritating	cannot be evaluated	irritating
6	non-irritating	non-irritating	non-irritating	irritating
7	non-irritating	non-irritating	non-irritating	irritating
8	irritating	moderately irritating	cannot be evaluated	irritating
9	non-irritating	corrosive / severely irritating	cannot be evaluated	non-irritating
10	strongly irritating	corrosive / severely irritating	corrosive / severely irritating	irritating
11	strongly irritating	corrosive / severely irritating	corrosive / severely irritating	irritating
12	non-irritating	non-irritating	non-irritating	irritating
13	non-irritating	mild irritating	non-irritating	irritating
14	irritating	corrosive / severely irritating	cannot be evaluated	irritating
15	mildly irritating	moderately irritating	cannot be evaluated	irritating
16	non-irritating	non-irritating	non-irritating	non-irritating
17	non-irritating	mildly irritating	non-irritating	non-irritating
18	non-irritating	moderately irritating	non-irritating	irritating
19	irritating	corrosive / severely irritating	cannot be evaluated	irritating
20	mildly irritating	mildly irritating	cannot be evaluated	irritating
21	irritating	moderately irritating	cannot be evaluated	irritating
22	irritating	corrosive / severely irritating	cannot be evaluated	irritating
23	non-irritating	moderately irritating	non-irritating	irritating
24	non-irritating	mildly irritating	non-irritating	irritating
25	non-irritating	moderately irritating	non-irritating	irritating
26	mildly irritating	corrosive / severely irritating	non-irritating	irritating
27	irritating	corrosive / severely irritating	cannot be evaluated	irritating
28	non-irritating	mildly irritating	non-irritating	irritating
29	mildly irritating	corrosive / severely irritating	cannot be evaluated	irritating
30	mildly irritating	corrosive / severely irritating	cannot be evaluated	irritating

5 CONCLUSION

In accordance with the set goals, the issue of using alternative biological models for testing eye irritation was studied in this dissertation. In the first part of the experimental work, a modification of the BCOP test, which uses bovine corneas as a test model, was designed and tested. This alternative test is considered a suitable alternative to eye corrosion and irritation testing, although it cannot be applied to all types of chemicals and mixtures. These limitations are mentioned not only in the literature, but especially in the OECD methodology. The experimental work was focused on solving the problem of a high number of false positive results in testing highly viscous and colored substances according to the classical procedure. For these substances, two modifications of the rinsing procedure were designed and validated in order to increase its efficiency.

Viscous substances tend to adhere to the surface of the cornea and thus reduce light transmission. A similar effect was observed for colored fabrics. The final modification of the washing system is based on a combination of gentle mechanical removal and extension of rinsing exposed corneas with the use of non-polar olive oil. The modification has proven to be very effective in removing adhesive samples and could potentially expand the scope of the BCOP test. The use of the treatment of the washing process is simple, cheap and does not require any special equipment and does not significantly increase the time. It is currently introduced into the SLP system and is used as standard in eye irritation testing.

The second part of the experimental work was focused on comparing the sensitivity of three selected alternative methods to the classical in vivo test in rabbits. The BCOP, RhCE and HET-CAM tests, which use completely different biological models, were chosen. The RhCE test, based on a 3D model of human keratinocytes, which is structurally similar to the surface part of the cornea, proved to be highly sensitive without the possibility of distinguishing the degree of irritation of chemicals. The HET-CAM test uses a highly perfused CAM of fertilized hens' eggs. This method was expected to have a high potential in the field of identification of mildly irritating substances, which was experimentally confirmed. The BCOP test using isolated bovine corneas has a good predictive power for severely irritating and corrosive substances as well as for non-irritants compared to the in vivo test in rabbits. Substances with the potential to cause mild or moderate eye irritation cannot be identified by this test.

A pair of alternative BCOP and RhCE tests are used for chemicals. The combination of the two models for the human cornea cannot cover all the mechanisms of action and thus completely replace eye irritation testing in animals. The inclusion of the HET-CAM method in the strategy of testing eye irritation with the BCOP test thus seems to be a possible variant to the current testing strategy recommended by the legislation. This test would be able to identify substances with the potential to cause mild or moderate eye irritation and classify them. Its successful validation and possible change in the method of lesion evaluation could lead to the legislative incorporation of this test into the group of tests for the classification of eye irritation and subsequent inclusion in the testing strategy. As a stand-alone test, HET-CAM has great potential in the area of mildly irritating substances such as cosmetics. The results confirmed that only a combination of differently sensitive in vitro tests using different biological models can completely replace the classic in vivo animal eye irritation test.

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