# USING THE HET-CAM TEST IN DETERMINING THE EYE IRRITATION POTENTIAL OF SOME PESTICIDES

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**Abstract**

Before being registered, pesticides must pass a number of toxicological tests. Examining the potential for eye irritation is one of these tests. The Draize test (*in vivo*), which covers the full irritation potential, is one of the most criticized methods due to the harm done to the test animals. To replace *in vivo* testing for eye irritation, a number of *in vitro* techniques, such as the hen's egg test–chorioallantoic membrane (HET-CAM), have been utilized to examine the toxicity of suspected irritants. In the HET-CAM test, pesticides are applied directly to the hen's egg chorioallantoic membrane, and the incidence of lysis, haemorrhage, or coagulation in response to the pesticide is observed for 5 minutes. In our study, a group of four pesticides were subjected to screening in order to establish *in vitro* data (HET-CAM) and compare with already established *in vivo* (Draize) findings. The findings showed a significant correlation of 75% between the HET-CAM and the *in vivo* United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) classification of the pesticides, and a 25% overprediction by the HET-CAM as compared to the *in vivo* data. The HET-CAM test can be said to be a good tool for examining the possible eye irritation potential of pesticides, which

can be suggested as a component of a series of experiments meant to lessen the use of mammals as test subjects and alleviate or completely do away with the sufferings that experimental animals endure.

Keywords: HET-CAM, eye irritation, *in vivo, in vitro*, UN GHS

**Összefoglalás**

A növényvédő szerek engedélyezését számos toxikológiai vizsgálat előzi meg. Egyik ezek közül a szemirritációs tulajdonságok megállapítására irányul. A korábban egyedüliként elfogadott Draize-teszt (*in vivo*), amely a teljes irritációs potenciált lefedi, a legtöbbet kritizált módszer. Helyettesítésére napjainkban több *in vitro* módszert is alkalmaznak, mint például a tyúktojás chorioallantois membránját felhasználó tesztet (HET-CAM). Vizsgálatunkban 4 peszticiden végzett HET-CAM teszt eredményeit hasonlítottuk össze az *in vivo* Draize eredményekkel. Az eredmények szignifikánsak, 75%-os korrelációt mutattak. A HET-CAM teszt alkalmas egy kísérletsorozat részeként a szemirritáció megállapítására.

Kulcsszavak: HET-CAM, szemirritáció, *in vivo*, *in vitro*, UN GHS

**Introduction**

According to the universal law of cause-and-effect, there is always a corresponding reaction to every action, this is also true for the use of agricultural pesticides, which aside from impacting the target also affect both the person applying them and the environment, depending on their toxicity level. As a result, determining the toxicity levels of these pesticides to ensure that they are within certain permissible limits is an important part of their approval process. In fact, REACH (Registration, Evaluation, and Authorization of Chemicals in the EU), for instance, stipulates that all pesticides must be tested for their level of toxicity before they can be used in

the European Union (European Commission, 2009). Primarily, the toxicity of pesticides over the past sixty years was investigated using the Draize test (Draize et al., 1944), which measures the eye irritation potential of the test item by placing it on the eyes of albino rabbits. Historically, one of the earliest public awareness raised about the Draize test came in 1980 via one of Henry Spira's New York Times advertisements captioned "How many rabbits does Revlon blind for beauty's sake?" which was met with harsh criticisms from animal rights activists who condemned the procedure and advocated for its replacement owing to the irreversible effects it has on the test items (Prinsen et al., 2017). It became critical in the years that followed to promote the development of non-animal test methods (*in vitro*) to replace the current use of animals (*in vivo*) in research (Choksi et al., 2019). Arguably, no other area of *in vitro* toxicology testing has compelled academic, governmental, and commercial efforts to create substitutes like the eye irritation test (Barile, 2010). Owing to this, several different tests have been devised to replace the use of rabbits in determining a chemical's propensity for causing eye irritation. When a test item is applied to the eye's anterior surface, it can cause alterations that are totally reversible within 21 days, this is called "eye irritation" (OECD 2019). One of the eye irritation tests involves observing the occurrence of negative changes (lysis, haemorrhage, or coagulation), that take place in the chorioallantoic membrane (CAM) of the hen egg (HET-CAM test) after exposure to test substances. It is possible to identify compounds that have the potential to irritate the eyes. Technically speaking, CAM is a complete tissue that includes arteries, capillaries, and veins. It reacts to harm by going into a full inflammatory mode, much like the tissue in a treated rabbit eye in the Draize test (Tavaszi and Budai, 2007). The goal of this study was to assess the use of the HET-CAM test in comparison with the recognized *in vivo* test for primary eye irritation with some pesticides in Hungary. To accomplish this, we used the HET-CAM assays to determine and compare the eye irritancy potential of some pesticides in Hungary.

## Material and method

Four pesticides were investigated in this study and applied in their original form and concentration, with characteristics described in the Table 1.

*Table 1 Investigated pesticides*

| Product Type | Product name | Physical characteristics | Active ingredients | Concentration |
|---|---|---|---|---|
| Herbicide | Viballa | Liquid | Halauxifen-metil | 3.0 g/L |
| Herbicide | Esteron 60 | Liquid | 2,4-D acid (2-ethyl-hexyl ester) | 600 g/L |
| Fungicide | Metkon 60 | Liquid | Metconazole | 60 g/L |
| Insecticide | Sivanto Prime | Liquid | Flupyradifurone | 200g/L |

The HET-CAM test was conducted according to (Luepke and Kemper, 1986), fresh fertile White Leghorn chicken eggs obtained from Gallus Kft within the weight range of 50 and 60 g were used for the test. Prior to the treatment, the fertile eggs were placed in a Ragus-type incubator, which was regulated to a temperature of between 37-38 °C and a relative humidity of between 60 -70%. The daily rotation of the incubator kept the embryos from adhering to the eggshell. On the ninth day of incubation, the eggs were candled, and the defective ones were discarded. Viable eggs were replaced in the incubator with the large ends positioned upward. On the tenth day, they were prepared for analysis, and a marker was used to mark the air cell portion. With the aid of a tapered scissors, the shell fragment above the air cell was cut off after the membrane had been gently moistened with a 0.9% NaCl solution. Using tapered forceps, the membrane was carefully removed without damaging the underlying blood veins. Only eggs that had a clearly defined fine vascular system on the CAM were used for the testing. The chorioallantoic membrane was exposed to 0.3 ml of the undiluted test material (pesticides), and the irritant effect of the test material was assessed by observing the occurrence of three endpoints for five minutes: lysis (vascular disintegration), haemorrhage (vessel bleeding), and coagulation (protein denaturation intra- and extravascular). Each endpoint's appearance time

was measured and recorded in seconds. Six eggs were examined with each test material in four replicas.

The data were analysed with the aid of a computer software and the pesticides classified following the notation proposed in (Invittox, 1990).

The computer program computes the irritant index (RI) using:

RI=(301-secH)/300×5 + (301-secL)/300×7 + (301-secC)/300×9

Where

RI = irritant index Sec= time in seconds H = haemorrhage

L=vascular lysis C= coagulation

According to their Irritation index, the tested pesticides were divided into three categories as shown in the Table 2.

*Table 2 HET-CAM classification (Invittox, 1990)*

| **Irritation index** | **Irritation category** |
|---|---|
| 0-0.9 | Not irritant |
| 1-8.9 | Irritant |
| 9-21 | Severely irritant |

## Results

Table 3 shows the findings of the HET-CAM test in comparison to the *in vivo* UN GHS classification of the pesticides.

*Table 3 In vitro and in vivo data*

| Product name | Lysis occurrence (seconds) | Haemorrhage occurrence (seconds) | Irritation index | Irritation category (HET-CAM) | *In vivo* UN GHS classification |
|---|---|---|---|---|---|
| Viballa | 13 – 17 | 60 - 90 | 10.48 | Severely irritant | Severely irritant |
| Esteron 60 | 175 – 190 | - | 2.77 | Irritant | Irritant |
| Metkon 60 | 10 – 15 | 85 - 120 | 10.3 | Severely irritant | Severely irritant |
| Sivanto Prime | 21 – 30 | 50 - 70 | 10.49 | Severely irritant | Irritant |

*(UN GHS - United Nations Globally Harmonized System of Classification and Labelling of Chemicals)*

The following deductions were made from Table 3:

Vascular lysis occurred between 13 and 17 seconds in eggs treated with Viballa, while haemorrhage occurred between 60 and 90 seconds and was classified as a severe irritant.

For eggs treated with Esteron 60, only vascular lysis was observed within 175 and 190 seconds of application, and therefore it was grouped as an irritant.

During treatment with Metkon 60, blood vessels broke down between 10 and 15 seconds and bled between 85 and 120 seconds. This was considered to be very irritating.

Lastly, after treatment with the insecticide Sivanto Prime, the CAM showed lysis at 21 to 30 seconds of its application and haemorrhaged at 50 to 70 seconds; this means it induced a severe irritation.

## Discussion

*In vitro* and *in vivo* data agreed 75% of the time (Table 3). Viballa, Esteron 60, and Metkon 60 had the same eye irritation classification in both tests, but Sivanto Prime was overestimated as a severely irritating substance in the HET-CAM test compared to the *in vivo* UN GHS classification. From our study, there was a strong correlation between the outcomes of the HET-CAM test and the *in vivo* data, which agrees with (Tavaszi and Budai, 2006; Kormos et al., 2009; Talaei et al., 2020 and Budai et al., 2021), the HET-CAM test showed a comparatively high level of accuracy and consistency with the *in vivo* method's results.

Even though it is subjective, the HET-CAM test is a good way to test how likely it is that pesticides will irritate the eyes (Tavaszi and Budai, 2006). It could be used as part of a series of experiments meant to reduce the use of mammals as test subjects and reduce or eliminate the pain and suffering that these animals go through.

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