

## **COMPARATIVE STUDY WITH A SET OF PESTICIDES TO ESTABLISH EYE IRRITANCY WITH HET-CAM AND DRAIZE TEST**

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### ***Abstract***

The chorioallantoic membrane (CAM) of chick embryo has been used extensively for many years in various fields of biological research, including virology, bacteriology and toxicology. The CAM is a complete tissue that responds to injury with a complete inflammatory reaction, this process similar to that induced by chemicals in the conjunctival tissue of the rabbit eye.

A possible model for assessing the irritation potential of a chemical or product to such a vascularized tissue is the chorioallantoic membrane of the embryonated hen's egg, as this is a highly vascular, thin membrane with relatively easy access for both treatment and assessment.

In recent years various *in vitro* methods have been developed to replace the heavily criticized Draize rabbit eye test for irritation testing.

One of the most studied alternative methods is the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM).

In our studies a comparative screening was done with a set of pesticides to establish parallel data on *in vitro* (HET-CAM) and *in vivo* (Draize) results.

In most cases good correlation was found between the HET-CAM assessment and results from the Draize rabbit eye test. The actual form of the HET-CAM test is a valuable prescreen for predicting ocular irritation potential of chemicals, and can be used to reduce the number of experimental animals. The HET-CAM test is useful as a part of a battery of tests to replace the Draize rabbit eye test.

**Keywords:** chorioallantoic membrane, *in vitro*, rabbit, Draize

### *Összefoglalás*

A tyúktojás chorioallantois membránját évek óta használják különböző tudományok biológiai kutatásaiban, úgymint a virológiában, bakteriológiában és toxikológiában. A membrán a nyúl szeméhez hasonlóan reagál a különböző vegyi anyagok okozta káros irritációs hatásokra.

Alkalmas modellnek ígérkezik az embrionálódott tyúktojás chorioallantois membránja az irritációs potenciálok meghatározásában, mivel rendkívül könnyen kezelhető és értékelhető.

Az elmúlt években számos alternatív módszert dolgoztak ki a sokat bírált Draize-féle primer szemirritációs vizsgálat helyettesítésére. Ezen alternatív technikák közül az egyik legtöbbet tanulmányozott, a tyúktojás chorioallantois membránját felhasználó (HET-CAM) teszt.

Vizsgálataink néhány peszticid *in vitro* és *in vivo* eredményeinek összehasonlítására irányultak.

A legtöbb esetben jó korrelációt találtunk a két teszt értékei között, azonban jelenlegi állapotában még nem jelenthető ki, hogy a HET-CAM teszt a Draize-féle primer szemirritációs vizsgálat kiváltására alkalmas lenne. Jelenleg, mint elővizsgálati módszer alkalmazható a kísérleti állatok számának csökkentésére.

### ***Introduction***

Pesticides must undergo numerous toxicological tests before registration. To determine the ocular irritation, only the Draize rabbit eye test is accepted now, which is one of the most criticized methods because of the pain inflicted on the test animals. Several *in vitro* methods have been used to investigate the toxicology of potential eye irritants with a view to replacing *in vivo* eye irritation testing. Now most of these methods are in process of validation.

The HET-CAM test, using the chorioallantoic membrane (CAM) of the chicken egg, is one of the possible suggested alternatives (Walum et al., 1992). In the HET-CAM test the chemicals are placed in direct contact with chorioallantoic membrane of the hen's egg. The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique as an indication of the likelihood that a chemical would damage mucous membranes (especially the eye) *in vivo*. The CAM is a complete tissue including arteries, capillaries and veins, and is technically easy to study (Leighton et al., 1985). It responds to injury with a complete inflammatory reaction, similar to the tissue of the rabbit eye.

In our studies comparative screening was performed with a set of pesticides to establish parallel data on *in vitro* (HET-CAM) and *in vivo* (Draize) results. In our experiment nine pesticides were evaluated by the HET-CAM test. The results were compared with *in vivo* data from Draize eye irritation test.

## ***Materials and methods***

### **Test materials**

The test preparations were included Total (480 g/l glyphosate), Orius 20 EW (200 g/l tebuconazole), Systhane Duplo (240 g/l myclobutanil), Domark 10 EC (100 g/l tetraconazole), Bumper 25 EC (250 g/l propiconazole), Megatox 40 EC (400 g/l chlorpyrifos-methyl), Glyphogan 480 SL (480 g/l glyphosate), Vertimec 1.8 EC (1.8 % abamectin), Glialka Star (441 g/l glyphosate). Pesticides were tested in all cases at 100% concentrations.

### **Methods**

#### **HET-CAM Test**

Shaver Rusticbrow chicken eggs were used. Before testing eggs were candled and discarded any which were defective. The eggs were incubated in a Ragus incubator. The temperature was 37.8°C and relative humidity was 70%. Eggs were rotated for 8 days to prevent the attachment of the embryo to one side of the egg. They were again candled on the 9<sup>th</sup> day and discarded any non-viable. The eggs were replaced into the incubator with the large end upwards but did not rotate, this was ensuring accessibility to the chorioallantoic membrane. On the 10<sup>th</sup> day they were prepared for assaying. The air cell was marked and the section of shell was removed with scissors. The membrane was moistened carefully with 0.9% NaCl solution and eggs were replaced in the incubator until ready for assaying.

Standards and test materials were prepared directly before each assay.  
Standards: 2 eggs with 1% Sodium dodecil sulphate and 0.1 M NaOH.  
Controls: 2 eggs with 0.9% NaCl.

Test: 6 eggs/group were treated with pesticide.

All of test solutions were run on 4 separated replicates.

The membrane was removed carefully with tapered forceps. A volume of 0.3 ml of test pesticide was added to the chorioallantoic membrane and the effect was observed over a period of 5 minutes. Haemorrhage, vascular lysis or coagulation can be seen on the chorioallantoic membrane. The starting time of reactions were recorded in seconds. A computer software was used to evaluate data (Invitox Protocol No. 47.).

The computer software uses the following algorithm:

$$RI = \frac{301-\text{secH}}{300} \times 5 + \frac{301-\text{secL}}{300} \times 7 + \frac{301-\text{secC}}{300} \times 9$$

Where H = haemorrhage, L = vascular lysis, C = coagulation, RI = irritation index, and  
sec = start second.

The classification categories based on irritation index are presented in Table 1.

*Table 1. Classification categories of HET-CAM test*

<b>Irritation index</b>	<b>Irritation category</b>
<b>0-0.9</b>	no irritation
<b>1-4.9</b>	weak irritation
<b>5-8.9</b>	moderate irritation
<b>9-21</b>	severe irritation

### **Draize Rabbit Eye Test**

In our experiment 3 New-Zealand albino rabbits were used in each assay. Based on the international guidelines (OECD Guidelines for Testing of Chemicals, Number 405, 2002), a separate control group was not necessary, the untreated eye serves as control. Rabbits were kept in individual cages of a climatic animal room. The temperature was 22-25 °C and relative humidity was 50-70%. Laboratory rabbit diet as food and tap water to drink were served *ad libitum*.

A volume of 0.1 ml of the pesticide was instilled into the conjunctival sac of each rabbit. Test solutions were prepared before each assay.

Ocular irritation was evaluated at 1 hour, 1, 2, 3, 4 and 7 days post instillation (Draize et al., 1944). Individual scores were recorded for each animal. The time interval with the highest mean score (Maximum Mean Total Score - MMTS) for all rabbits was used to classify the test substance.

The classification categories according to MMTS are shown in Table 2.

*Table 2. Classification categories of Draize rabbit eye irritation test*

<b>MMTS</b>	<b>Irritation classification</b>
<b>0-19</b>	no irritation
<b>20-49</b>	moderate irritation
<b>50-79</b>	severe irritation
<b>80-110</b>	super irritation

### ***Results***

#### **Results of the HET-CAM Test**

The numerical data are summarised in Table 3.

After the treatment with Total first vascular lysis was occurred between 9 and 26 sec, followed by haemorrhage that was observed from

11 to 200 sec. Total was a severely irritative pesticide with irritation index of 10.29.

After the treatment with Orius 20 EW vascular lysis was recorded from 20 to 37 sec and the haemorrhage was noted from 28 to 50 sec. On the base of irritation index (10.79) Orius 20 EW was severely irritative.

After instillation of Systhane Duplo vascular lysis was observed between 12 and 20 sec, and followed by haemorrhage from 40 to 80 sec. The test material has severe irritative potential according to the irritation index (10.76).

After the treatment with Domark 10 EC vascular lysis was started in 20<sup>th</sup> sec and haemorrhage was occurred in 90<sup>th</sup> sec. The irritation index showed that Domark 10 EC was severe irritant (9.62).

The treatment of eggs with Bumper 25 EC resulted in vascular lysis between 50 and 60 sec, followed by mild haemorrhage that lasted from 75 to 130 sec. Bumper 25 EC was a severely irritative pesticide with irritation index of 9.18.

After instillation of Megatox 40 EC vascular lysis was recorded from 20 to 37 sec, and the haemorrhage was observed from 45 to 140 sec. The test material had severe irritative potential according to the irritation index (10.4).

When Glyphogan 480 SL was used, vascular lysis was experienced between 15 and 25 sec. On the base of irritation index (6.55) Glyphogan 480 SL was moderately irritative.

The Vertimec 1.8 EC caused vascular lysis from 10 to 40 sec and haemorrhage was occurred from 60 to 70 sec in some eggs. The test material resulted in moderately irritative potential according to the irritation index (6.45).

After instillation of Glialka Star vascular lysis was observed from 9 to 35 sec. The irritation index showed that Glialka Star was moderately irritant (6.67).

*Table 3. Irritation indices from HET-CAM test*

Test materials	Irritation index
<b>Total</b>	10.29
<b>Orius 20 EW</b>	10.79
<b>Systhane Duplo</b>	10.76
<b>Domark 10 EC</b>	9.62
<b>Bumper 25 EC</b>	9.18
<b>Megatox 40 EC</b>	10.4
<b>Glyphogan 480 SL</b>	6.55
<b>Vertimec 1.8 EC</b>	6.45
<b>Glialka Star</b>	6.67

### **Results of the Draize Rabbit Eye Test**

The data are presented in Table 4.

After the instillation of Total followed by positive conjunctival responses with severe redness, severe chemosis and strong discharge up to day 2 after treatment. Responses turned slight from day 3 after instillation. There was no corneal reaction and iritis. On the base of irritation index (22) Total was a moderately irritative in rabbits.

Severe redness, severe chemosis and strong discharge were noted for 3 days post instillation of Orius 20 EW. The observations were reduced to the 4<sup>th</sup> day. On the cornea moderate opacity was noted from 1<sup>st</sup> day, which was observed during the whole observation period. Iritis was occurred from the 1<sup>st</sup> day and it was permanent during the whole observation period. Orius 20 EW was a severely irritative pesticide with irritation index of 66 in rabbits.

Severe redness, severe chemosis and strong discharge were observed for 3 days post instillation of Systhane Duplo. Responses turned slight from day 4 after instillation. Moderately corneal opacity and



slight iritis were noted in two rabbits. Slight corneal opacity and severely iritis were observed in one rabbit. The test material had severely irritative potential according to the irritation index (52) in rabbits.

The instillation of Domark 10 EC caused positive conjunctival responses with severe redness, severe chemosis and strong discharge up to day 4 after treatment in two rabbits. In one rabbit severe conjunctival responses were observed that lasted for day 1. On the cornea moderate opacity was noted from the 1<sup>st</sup> day until the end of the observation period in two rabbits. Iritis was noted on the 1<sup>st</sup> day that did not return to normal in the case of two rabbits. On the base of irritation index (48) Domark 10 EC was a moderately irritative in rabbits.

The treatment with Bumper 25 EC resulted in slight redness, slight chemosis and slight discharge to the 2<sup>nd</sup> day. Iritis was not recorded. On the cornea no opacity was noted during the whole observation period. Bumper 25 EC was not irritative pesticide with irritation index of 11 in rabbits.

Severe redness, severe chemosis and strong discharge were observed for 4 days post instillation of Megatox 40 EC in one rabbit. Slight redness, chemosis and discharge were noted up to day 3 after treatment in two rabbits. The observed symptoms were returned to normal by day 4. Iritis was not seen. On the cornea opacity was observed in one rabbit. On the base of irritation index (22) Megatox 40 EC was a moderately irritative in rabbits.

The instillation of Glyphogan 480 SL induced positive conjunctival responses with moderate redness, severe chemosis and strong discharge up to day 4 after treatment. Iritis was not observed. On the cornea moderate opacity was noted from the 1<sup>st</sup> day and strong opacity from the 4<sup>th</sup> day that lasted to the end of observation period. On the base of irritation index (65) Glyphogan 480 SL was a severely irritative in rabbits.

The treatment with Vertimec 1.8 EC resulted in moderate redness, chemosis and discharge to the 1<sup>st</sup> day in one rabbit. In other rabbits these symptoms were observed to the 4<sup>th</sup> day. Iritis was occurred only in 1 hour. Weak corneal opacity was noted from the 1<sup>st</sup> day until the end of the observation period, but this alteration was moderate in one rabbit from the 3<sup>rd</sup> day. Vertimec 1.8 EC was moderately irritative pesticide with irritation index of 46 in rabbits.

After the treatment with Glialka Star moderate conjunctival responses were recorded to the 2<sup>nd</sup> day. The iris and the cornea did not change. The eyes returned to normal in 3<sup>rd</sup> day. The test material had moderately irritative potential according to the irritation index (21) in rabbits.

*Table 4.*  
*Draize irritation indices of test materials corresponding*  
*irritation categories*

<b>Test materials</b>	<b>Irritation index</b>
<b>Total</b>	22
<b>Orius 20 EW</b>	66
<b>Systhane Duplo</b>	52
<b>Domark 10 EC</b>	48
<b>Bumper 25 EC</b>	11
<b>Megatox 40 EC</b>	22
<b>Glyphogan 480 SL</b>	65
<b>Vertimec 1.8 EC</b>	46
<b>Glialka Star</b>	21

### ***Discussion***

The HET-CAM test has the upper edge on *in vivo* methods. It is faster, cheaper and simpler than the Draize rabbit eye test. Several substances and formulations have been tested by HET-CAM and a good correlation has been found between the HET-CAM results and data based on the Draize eye test (Leighton et al., 1985; Luepke and Wallat, 1987; Sina et al., 1995; Spielmann et al., 1996; Budai and Várnagy, 2000; Tavaszi and Budai, 2006; Tavaszi et al., 2008; Budai et al., 2010.).

In our study, a good correlation was seen between the data from *in vitro* and *in vivo* methods (Table 5). However, based on the results, the HET-CAM test gave the same irritancy category for four pesticides (Orius 20 EW, Systhane Duplo, Vertimec 1.8 EC, Glialka Star). Based upon the comparison of *in vitro* and *in vivo* tests, the HET-CAM test showed a lower irritation potential for one pesticide (Glyphogan 480 SL) and a more severe irritation potential for four products (Total, Dömark 10 EC, Bumper 25 EC, Megatox 40 EC). The drawbacks are: it is subjectivity, it can not be used in case of colored materials because they hide the membrane and the testing of solid materials is circumstantial. The results of the investigated agricultural materials with HET-CAM test achieved similar results to the Draize rabbit eye test.

*Table 5.*  
*Irritation categories by HET-CAM test in comparison to*  
*irritation categories by Draize rabbit eye test*

<b>Test materials</b>	<b>Category from HET-CAM test</b>	<b>Category from Draize rabbit eye test</b>
<b>Total</b>	severe irritation	moderate irritation
<b>Orius 20 EW</b>	severe irritation	severe irritation
<b>Systhane Duplo</b>	severe irritation	severe irritation
<b>Domark 10 EC</b>	severe irritation	moderate irritation
<b>Bumper 25 EC</b>	severe irritation	no irritation
<b>Megatox 40 EC</b>	severe irritation	moderate irritation
<b>Glyphogan 480 SL</b>	moderate irritation	severe irritation
<b>Vertimec 1.8 EC</b>	moderate irritation	moderate irritation
<b>Gialka Star</b>	moderate irritation	moderate irritation

Today there is still insufficient comparative data available using methods based on the chorioallantoic membrane of hen's egg to be an alternative to Draize rabbit eye test. As a result we need to involve further agricultural chemicals and keep on testing.

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