HET-CAM TEST

The potential irritancy of compounds may be detected by observing adverse changes which occur in the chorionallantoic membrane of the egg after exposure to test chemicals.

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Rationale

Chemicals are placed directly onto the chorionallantoic 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 potential of a chemical to damage mucous membranes (in particular the eye) *in vivo*.

Basic Procedure

Hen's eggs are rotated in an incubator for 9 days after which time any defective eggs are discarded. The shell around the air cell is removed and the inner membranes are extracted to reveal the chorionallantoic membrane. Test chemicals are added to the membrane and left in contact for 5 minutes. The membrane is examined for vascular damage and the time taken for injury to occur is recorded. Irritancy is scored according to the severity and speed at which damage occurs.

Critical Assessment

The test has several advantages including its simplicity, rapidity, sensitivity, ease of performance and its relative cheapness.

A factor to consider is the fertility and the ability of the eggs to hatch. The survival of chickens is dependent on a complex interrelationship of ecological factors (e.g. the genetic background and the age of the mated birds, the nutritional status and general management of the flock, and in part seasonal variations). Eggs should, therefore, be obtained from reliable local contractors. (The authors have produced some empirical data on the fertility of the particular flocks they use. The fertility of middle-aged flock is approximately 90% with 10-15% defective eggs. On average there are 20% lesions produced during preparation.)

The major disadvantage of the procedure is the subjective nature of the evaluation of the results. This is overcome to a certain extent by the inclusion of positive standards and by using a comprehensive scheme for scoring the irritant effects of the chemicals.

The exposure period of 5 minutes to the test chemical has been found to be sufficient to reveal irritant/toxic effects (longer exposure does not appear to yield any additional information).
A factor for consideration is whether the Hen's egg test may be considered as an animal experiment. At present the test is often looked upon as being borderline, although it has potential to be used in a manner likely to reduce the number of mammals used in conventional testing and also to contribute towards a reduction in the associated suffering.

Test Status

This test, along with several other *in vitro* systems, is presently undergoing validation as an alternative test to replace the Draize Rabbit Eye Test, in a national interlaboratory study started in June 1988, by the Federal Health Office (BGA) of the Federal Republic of Germany (FRG).

The aim of this collaborative study is to validate the classification of chemicals, with regard to their irritation potential, using the Neutral Red/ Kenacid Blue (NR/KB) cytotoxicity assay and the Hen's Egg Test Chorioallantoic Membrane (HET-CAM) assay according to Lupke. The FRG Public Health Office (BGA) is coordinating the scheme which includes, 12 toxicology laboratories in the chemical industry, universities, the BGA and other research institutions who will study 44 substances with a variety of chemical, biochemical, and toxicological characteristics. The validation test is intended to provide comparative data for the development of an alternative routine test scheme, and which is performed under routine laboratory conditions.

The validation project of alternatives for the Draize test consists of three parts: a preliminary phase, an interlaboratory assessment, and, finally, the development of a database of results. During the preliminary phase the cytotoxicity test and the HET-CAM assay have been established in the different laboratories. The participants have agreed on standard and mandatory protocols and on the choice of chemicals. Two preliminary trials have been performed with 4 test substances.

During the interlaboratory assessment 35 chemical substances of a variety of chemical structure groups have been tested with both alternative techniques in 12 laboratories under conditions that will be defined in the preliminary phase of the study. The main purpose of the validation phase is the comparative and statistical evaluation of all data at the BGA followed by a final scientific validation which could prove of interest to regulatory authorities. This assessment determines both the reproducibility of the results within a given laboratory and of a given test between laboratories.

Preliminary findings indicate that data from the HET-CAM test appears to correlate better than the two cytotoxicity tests when compared to *in vivo* Draize scores. The cytotoxicity tests give a greater number of false positives and negatives compared to the HET-CAM test. The cytotoxicity tests have, however, given better reproducibility of test data, within and between laboratories, than the HET-CAM. This is most probably due to the automated determination of NR and KB values and to the highly subjective determination of the toxicological endpoints in the HET-CAM test which are difficult to standardize. In conclusion, both the cytotoxicity tests and the HET-CAM test can provide reproducible results if carried out under routine conditions with well trained operators.

The third phase of the validation project, database development, commenced on June 1st, 1990. Seven laboratories are testing a total of 200 chemicals which again include a variety of chemical classes.

Chemicals Tested

Acetone	Lactic acid (5%)
Acetonitrile	*n*-Hexane
Acrylamide	Nicotinamide
Aniline	Nitrobenzene
Ascorbic acid	Phenol
Benzalkonium chloride (0.5%)	Propanediole
Benzoic acid	2-Propane-1-ol
2-Butoxyethanol	Pyridine
Copper(*II*) sulphate	SDS (1%)
Cyclohexanol	Sodium chloride
DEHP (100%)	Tetrachloroethane
Dimethylsulphoxide	Thiourea
EDTA-Na salt	Toluene
Ethanol	

References

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Toxic. in Vitro, **5 No.5/6**, 539-542.

Procedure Details

Note: The herewith included details on the procedure have been sent to the person responsible for the method to update or confirm it. As soon as new information will become available this version will be updated.

Animal
White Leghorn chicken eggs (Shaver Starcross 288A)
The White Leghorn chicken has been selected for several reasons;
The ability to hatch the eggs of this breed is very consistent and reproducible. There does not appear to be any hereditary defects in this

breed.

Equipment

Incubator with an automatic rotating device, e.g. Ehret GmbH,
220 V, 50 Hz, 360 watt
Optimum temperature: 37.5^{0}C (± 0.5^{0}C)
Relative humidity 62.5% (± 7.5%)

Candling light
Dentist's rotating sawblade
Computer with appropriate software (HET-CAM evaluation program) - not commercially available (authors will give assistance to interested scientists)
Cold-light lamp
pH-meter
Thermometers
Tapered forceps
Pipettes (300µl application)
Stopclock

Materials

NaCl
SDS
0.1 N NaOH

Make up the following solutions:
0.9% NaCl solution in distilled water
1% SDS solution in distilled water

Test chemicals
Make up the chemicals in 0.9% NaCl solution or olive oil.

Method

N.B. Avoid any shaking, unnecessary tilting, knocking, and all other mechanical irritation of the eggs when preparing them for the assay.

Incubation of eggs
Select fresh fertile 50-60g eggs. Candle the eggs and discard any which are defective.

Place the eggs flat onto incubator trays in a 37.5^{0}C incubator and rotate for 8 days to prevent the attachment of the embryo to one side of the egg.
Check the temperature and humidity at the same time each day.
Candle the eggs on the 9th day and discard any non-viable eggs.
Replace in the incubator with the large end upwards but do not rotate, thus ensuring accessibility to the chorionallantoic membrane.
On day 10 prepare the eggs for assaying.

Assay preparation
Candle each egg to ensure that all are viable. Use cold lamp to ensure an optimal illumination of the chorioallantoic membrane.

Carry out in a fume cupboard with safety goggles to prevent inhalation and contact with the fine egg shell powder. Mark the air cell using a rotating dentist-sawblade and pare the section of the shell off.

Carefully moisten the membrane with 0.9% NaCl solution at 37^0C.

Replace eggs in incubator until ready for assaying (maximum of 30 minutes between opening the eggs and starting the assay).

Freshly prepare standards and test solution (in the appropriate solvent) before each assay at room temperature. Measure and record ph.

Assay procedure
Take the opened egg out of the incubator, pour off the 0.9% NaCl solution, carefully remove the egg membrane without injuring any underlying blood vessels using tapered forceps.

Add 0.3ml of the standard, or test chemical solution to the CAM.

Observe the reactions on the CAM over a period of 5 minutes. Monitor the appearance of:

haemorrhage (Bleedings)
vascular lysis (Blood vessel disintegration)
coagulation (protein denaturation intra- and extra vascular)

Record in seconds, the time for each reaction to occur and calculate an **irritation score (IS)**.

$$IS = \frac{301 - sec\ H}{300} \cdot 5 + \frac{301 - sec\ L}{300} \cdot 7 + \frac{301 - sec\ C}{300} \cdot 9$$

H : Haemorrhage
L : Vessel lysis
C : Coagulation
sec : Start Second

When determining the threshold the degree of severity of each reaction after treatment time has to be recorded according to the following scheme:
0 = no reaction
1 = slight reaction
2 = moderate reaction
3 = severe reaction

The threshold is then defined to be the highest concentration, at which slight reactions occur. To determine the threshold apply 0.3ml of the starting concentration (a good choice is 5% if no further information is given) to three eggs each. Graduate the severity of the main reaction after 5 minutes. If the observed reaction is slight , double the concentration. If the reaction is moderate or severe, divide the concentration by two or ten to get the next test concentration. Proceed further until the threshold concentration is found.

Test Scheme
For a given chemical the procedure consists of four steps:
1) Determine the irritation score (IS) for the two standards with two eggs

each.

N.B. 1% SDS should give an IS of 10±2 and 0.1 NaOH an IS of 15±3.

2) Determine the threshold concentration of the test chemical as described above.

3) Determine the IS for a 10% solution for three eggs. For insoluble substances take the supernatant of a standard solution.

4) Determine the IS for the pure substance (100%). If the test chemical is an insoluble solid substance, proceed as follows: Instead of determining the IS, put some grains of the substance onto the CAM to cover approximately half of its surface. After 5 minutes carefully rinse off the test material with NaCl solution and record the severity of each of the three reactions (haemorrhage, lysis, coagulation) according to:
0 = no
1 = slight
2 = moderate
3 = severe

If any reaction of degree 3 has been observed, repeat the procedure with three new eggs, rinsing after one minute.

At the end of the assay kill the embryos as quickly as possible (e.g. by placing the eggs into a freezer at -20^{0}C).

Calculations and Classifications
Calculate the mean value of the IS for the three eggs for each of the two runs and both concentrations as well as the mean over both runs of the IS and threshold concentration.

A classification of the irritating potential can be carried out according to the following (preliminary) classification scheme.

Threshold (TH) concentration	Irritation score (10%)	Severity	Classification
TH < 1%			severe/corr
1.0 < TH < 2.5	> 16		severe/corr.
2.5 < TH < 10.0	< 16	severe reaction after 1 min.	severe/corr.
1.0 < TH < 2.5	< 16		irritant
2.5 < TH < 10.0	> 16		irritant
2.5 < TH < 10.0	< 16	severe reaction after 5 min	irritant
2.5 < TH < 10.0	< 16	weak or no reaction	moderate
10.0 < TH	> 16		moderate
10.0 < TH	< 16	severe reaction	moderate
10.0 < TH	< 10		no/slight

Experimental Data

Preliminary results of the interlaboratory study are published in:
Spielmann, H. *et al.* (1991): Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. *Toxic. in Vitro*, **5 No. 5/6** , 539-542.

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