Experience with the chick embryo chorioallantoic membrane (CAM) and isolated bovine eye as alternative test models for mucous membrane irritation

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Next to the LD_{50} acute toxicity test in routine toxicology, the Draize rabbit test for eye irritation is the most controversial routine animal testing procedure. The test procedure still in use for evaluating the eye irritant potential of chemicals was proposed more than 40 years ago by Draize, Woodard and Calvery (1944). The test is criticized for its lack of scientific value as much as for its questionable ethical acceptability. The rabbit eye is generally considered more sensitive to chemicals than the human eye although this is not always the case. In addition there are structural and physiological differences which make the rabbit eye an unsatisfactory model for the human eye. Moreover, the results on the same chemicals can vary widely from laboratory to laboratory and also within the same laboratory, mostly because of the subjective nature of the assessment of test results (Heywood, 1981).

Until recently there have been little attempt to develop an alternative to the Draize test. Simple cytotoxicity assays using established cell lines have been used with some success (Simons, 1980). Encouraging results have also been obtained with the chorioallantoic membrane of chick embryos (Luepke, 1985, Leighton et al., 1985). Guidelines for the toxicity testing of chemicals have also specified that chemicals may be tested in perfused isolated rabbit eye before tests in living rabbits. A promising test with isolated rabbit eyes has been proposed by Burton et al. (1981).

As in other fields of toxicology as for example mutagenicity and carcinogenicity, a non-animal alternative for the Draize test may well result in a battery of systems capable of predicting human eye irritancy more reliably.

At Scantox, Biological Laboratory Ltd. a strategy was layed down for development of alternative test systems which should include testing for mucous-membrane and corneal irritation potency. Prerequisites were that the tests should be sensitive, rapid, inexpensive and the test procedures should be simple to introduce in the laboratory. It was equally important that the test material should be readily available for any laboratory.

The chick embryo and isolated bovine eyes were natural test materials fulfilling the prerequisites. The chorioallantoic membrane of chick embryo could mimic the conjunctival mucous membrane whereas the isolated eye could be a corneal model.

Chorioallantoic membrane (CAM)-test

This test was adopted from Luepke (1985). White Leghorn eggs incubated for ten days were purchased from a local brooding-house and transported to the laboratory. The egg shell was scratched around the air cell with a dentist's rotary saw and then pared off. After careful removal of the inner egg membranes the vascular CAM was exposed.

The test substance, dissolved or suspended, was dropped onto the membrane in a volume of 0.2 ml. In the case of solid test materials, 0.1 g was applied on the CAM and irrigated after 20 sec with 0.9% saline at 37°C. For each test substance and each concentration three eggs were used. In the case the vehicle was known, three eggs treated with the vehicle only, served as control.

After application of the test substance, the chorioallantoic membrane, blood vessels and albumen were examined and scored for irritant effects (table 1) at 0.5, 2 and 5 min after application. The numerical time-dependent scores for hyperaemia, haemorrhages and coagulation were summed to give a single numerical

value indicating the irritation potential of the test substance on a scale with a miximum value of 21 (table 2).

Bovine eye irritation test

Bovine eyes from a local slaughterhouse were kept in 0.9% saline at 37°C until use. They remain viable for up to 4 hours. After examination for corneal damage (opacity after fluorescein), 4 eyes were equilibrated in a water bath at 37°C. A silicone ring was placed on the cornea for easy and reproducible test substance administration. The chemicals were applied to the cornea inside the ring. After 30 sec the eyes were rinsed with saline. The extent of corneal injury was assessed by evaluating the opacity followed by the application of flurorescein to examine the integrity of the corneal epithelium under U.V. light. An eye which was not responding to the application of test substance was checked for responsiveness afterwards with application of 70% ethanol and subsequent examination under U.V. light after flourescein application.

Table 1
Scoring scheme for irritation testing on chick chorioallantonic membrane

Effect	Score Time (min.) 0.5	2	5
Hyperaemia	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

Table 2
Classification of cumulative scores in CAM test

Cumulative score	Irritation classification
0-0.9	Practically none
1-4.9	Slight
5-8.9	Moderae
9-21	Strong

Depending on the results obtained, the test substance was categorized as not irritant, slightly, moderately or severely irritating.

Comments

The chorioallantoic membrane of the chick embryo is a complete tissue including arteries, capillaries and veins and it is technically easy to study. It responds to injury with a complete inflammatory reaction similar to that induced in the conjunctival tissue of the rabbit eye after exposure for the same time.

With the CAM-test only 0.1 g of solid test materials was applicated whereas dissolved or suspended test substances were applied in a volume of 0.2 ml. The reason for choosing only 0.1 g of solids was the risk that a larger amount by its localized weight could disrupt the membrane and cause harmorrhage.

The CAM-test is a very sensitive model. We have seen effects in the CAM-test with dissolutions which according to the rabbit eye model could not be characterized as eye irritant. The reproducability of the test seems satisfactory since we have obtained about the same result with the same dissolution on different testing days. However, it is our experience that the test is less usefull for crude substances and for pharmaceutical preparations with very high viscosity. They do not adequately spread in the layer of fluid on the membrane.

The bovine-eye results in our laboratory indicate that the eyes are viable for more than 4 hours after excision. However, they become probably less responsive with time. Death of the corneal epithelium is clearly demonstrated with fluorescein.

These two tests seem to be usefull as screening tests for evaluation of series of closely related molecules or products with respect for irritation potential. The tests may not substitute in vivo testing of the final product selected for marketing. For such a product in vivo confirmation of the screening test result may be required. However, distressing in vivo tests can be avoided because the product selected on basis of the screening tests most likely will be the

product with relatively low irritation potential also in in vivo tests.

Several factors have strengthened the development in in vitro toxicology. The most important factor is the advances and knowledge gained in the area of techniques combined with the recognition by the scientific community that these techniques may provide useful tools for safety assessment. Another factor is the pressure imposed on industries, regulatory agencies and academic community to replace or reduce the use of animals in research and routine testings. However, regulations often require specific animal tests for product approval. In addition, the possibility of litigation is a factor that imposes conservatism in the process of testing strategy decisions. Finally, these new methods require further validation and standardization before they can obtain general acceptance by scientific and regulatory communities. According to a recent OECD proposal validated in vitro methods may be accepted as the sole testing for eye irritation potential.

Sammendrag:
Den såkaldte Draize test til bedømmelse af kemiske stoffers øjenirritative virkning i kaniner er kontroversiel på grund af videnskabelige og etiske aspekter. Alternative metoder så som cytotoksicitet i cellekultur og irritativ virkning på fosterhinder af embryonerede hønseæg eller isolerede kaninøjne har været forsøgt. Metoder med embryonerede hønseæg og med isolerede okseøjne er søgt introduceret i laboratoriet ved Scantox A/S. Chorion-allantois membranen i 10 dage embryonerede hønseæg observeres for hyperæmi, blødning eller koagulation 0,5, 2 og 5 min after applikation af testpræparat. Forandringerne tildeles en værdi, der danner grundlag for en klassifikation af irritationens sværhedsgrad. Isolerede øjne fra nyslagtede kreaturer opbevares i varm fysiologisk saltvand indtil testning, som består i applikation af testpræparat i en på hornhinden anbragt siliconering. Ved hjælp af fluorescein og ultraviolet lys bedømmes den irritative virkningsgrad på hornhindeepithelet. Begge metoder er meget følsomme, og de synes at være anvendelige for serier af produkter. Deres særlige værdi ligger i anvendelse til selektion af produkter, som vil give færrest gener i en efterfølgende in vivo testning.

Yhteenveto / K. Pelkonken
Kanilla tehtävää Draizen testiä kemiallisten aineiden silmää ärsyttävän vaikutuksen tutkimiseksi on arvosteltu tieteellisten ja eettisten näkökohtien pohjalta. Testille on pyritty löytämään vaihtoehtoja, kuten sytokoksisuus soluviljelmssä, ärsyttävyys kanan sikiökalvoille sekä kanin eristettyjen silmien käyttö kananalkion sikiökalvoja ja eristettyjä naudan silmiä käyttävät menetelmät. Kymmenen päivän ikäisen kananalkion chorion-allantoiskalvoa tarkkaillaan verentungoksen, verenvoutojen tai sameenemisen havaitsemiseksi puolen, yhden ja kahden minuutin kuluttua testipreparaatin antamisesta. Muutokset arvioidaan ja tämän perusteella aineet voidaan jakaa luokkiin ärsyttävyyden perusteella. Toisessa menetelmässä käytetään teurastamolta saatuja eristettyjä naudansilmiä, joita pidetään lämpimässä fysiologisessa keittosuolaliuoksessa testaukseen asti. Testiaine annostetaan sarveiskalvolla olevan silikonirenkaan sisään. Ärsytysvaikutusta arvioidaan fluoreskeiinin ja ultraviolettivalon avulla. Molemmat menetelmät ovat hyvin toistettavia ja antavat käyttökelpoisia tuloksia useilla aineilla. Menetelmät ovat arvokkaita erityisesti siksi, että niiden avulla kyetään sulkemaan pois in vivo-testauksesta aineet, joilla voi olla voimakkaasti ärsyttävä vaikutus.

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