HET-CAM Bioassay as *In Vitro* Alternative to the Croton Oil Test for Investigating Steroidal and Non-steroidal Compounds

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**Summary**

In this study the irritation phenomena at the chorioallantoic membrane of incubated hen's eggs as an *in vitro* model (HET-CAM assay) were investigated in comparison to the *in vivo* croton oil test by including hydrocortisone, indomethacin, phenylbutazone, acetylsalicylic acid, rutin, quercetin, apigenin, and p-coumaric acid as steroidal and non-steroidal test substances. For the first time the two methods were compared in a valid way with the perspective of a realistic reduction of animal experiments. It should be investigated whether an *in vitro*-*-in vivo* correlation exists and, if there is any possibility, to replace the *in vivo* model by an *in vitro* test system. Both bioassays were able to demonstrate the anti-inflammatory potency of the constituents tested. The determination of the anti-inflammatory activity of all compounds in the two test systems showed individual trends of inhibitory effects. However, the *in vitro* HET-CAM test was much more sensitive in comparison to the *in vivo* croton oil test. The croton oil test gave dose-effect correlations in the anti-inflammatory substances investigated. The modified HET-CAM assay did not provide clear dose-effect ratios. The HET-CAM assay is an inexpensive test being easy to manage after a short practical training. Because of its sensitivity the HET-CAM assay could be considered a suitable tool for qualitative testing of the anti-inflammatory activity of substances if no appropriate dose-effect curves are required. From these results it can be concluded that the different courses of the dose-effect curves may be primarily due to different mechanisms of action.

**Keywords:** anti-inflammatory test systems, HET-CAM assay, croton oil test

1 Introduction

Pharmacological tests are essential for the development and approval of pharmaceutical drugs. For testing the anti-inflammatory activity animal models are used frequently. Animal experimentation is one of the most difficult ethical issues. Historically, the animal protection movement and the scientific community saw themselves as natural opponents in the battle over animal rights and the validity of animal tests. The search for animal alternatives began 30 years ago. Now research of alternative methods is widely perceived as offering animal welfare, scientific and often economic gains. Inflammation is a multifaceted process
provoked by irritating agents of physical, chemical or biological origin which affects the blood vessels and related tissue within the damaged area. In the present study the pharmacological actions of eight commercially available steroidal and non-steroidal compounds were evaluated using as alternative an in vitro test model, the modified hen's egg chorioallantoic membrane test (HET-CAM assay) of D'Arcy et al. (1967) in comparison to the croton oil test (Tubaro et al., 1985) as animal model. Croton oil is the main constituent of the seeds of Croton Tiglium (Euphorbiaceae) which is indigenous to the tropical areas of Western Africa and Asia. The yellow-brownish viscous oil consists of esters of the diterpene alcohol phorbol with different fatty acids. In former times croton oil was used as laxative but nowadays this application is obsolete because of its co-carcinogenic effect. After topical application phorbol esters induce strong inflammations with edema formation on skin and mucosa. Only a few publications report an in vivo-in viva correlation for the results achieved with the HET-CAM assay (Luepke et al., 1990a; Luepke, 1992). The HET-CAM assay serves also as an excellent system for studying the mechanisms of photodynamic therapy in tumors as well as in blood vessels (Roberts and Hasan, 1992). It is further used for testing the embryotoxicity (Luepke, 1982), vascular effects (Luepke et al., 1990b) and the quantification of the angiogenesis and the anti-angiogenesis (Nguyen et al., 1994). The HET-CAM assay as in vitro test is also replacing the Draize rabbit's eye test for irritancy testing (Spielmann et al., 1989). The CAM is a living vascularised membrane which can be used to determine vascular activity of substances. The irritation is induced by sodium dodecyl sulphate and the phenomena of irritation which is achieved by the pharmaceuticals is observed at the CAM after incubation. The croton oil test is based on the ability of a test substance to inhibit the croton oil induced dermatitis on the mouse ear after topical application.

This paper is a contribution to our continuing research on the application of different anti-inflammatory test systems on pure compounds and plant extracts (Polligger et al., 1999; Polligger et al., 2000). The aim of this study is to provide comparative data for the evaluation of the two test systems.

2 Animals, material and methods

Male CD 1 strain mice (weight 25-30g) were obtained from Charles River Calco, Italy.


Eight pure steroidal and non-steroidal compounds, hydrocortisone (Merck, Darmstadt/Germany; 1.24608), indomethacin (Fluka, Buchs/Switzerland; 57413), phenylbutazone (Sigma, Steinheim/Germany; P-8386), acetylsalicylic acid (Sigma, Steinheim/Germany; A 5376), rutin (Roth, Karlsruhe/Germany; 7176), quercetin (Fluka, Buchs/Switzerland; 83370), apigenin (Roth, Karlsruhe/Germany; 8728), and p-coumaric acid (Fluka, Buchs/Switzerland; 28200) were chosen as test samples. Croton oil (Fluka, Buchs/Switzerland; 28003) and sodium dodecyl sulphate (SDS; Merck, Darmstadt/Germany; 1.12012) were used as inflammatory agents and agarose (Merck, Darmstadt/Germany; 1.16802) as carrier in the HET-CAM test.

2.1 Croton oil test

The principle of the croton oil model is based on the application of 15 µl croton oil (5 µg/µl; dissolved in acetone) in combination with a test substance to the inner surface of the right ear (1cm²) of anaesthetised mice (Ketalar HCl; 145 mg/kg intraperitoneally). The animals remain in anaesthesia during 30 minutes. The other ear remained untreated. All experiments were started between 11.00 to 12.00 a.m. (Tubaro et al., 1985). The reference substances were dissolved in acetone. The tested drugs were applied at a dosage of 0.01-2.50 µM/ear starting with a stock solution (concentration 2.50 µM) which was subsequently diluted. Six hours after the application the mice were killed and a plug was taken from each treated and untreated ear. The difference in weight between the two plugs is

Fig. 1: HET-CAM assay. Inhibition of the inflammation of the hen's egg chorioallantoic membrane (CAM) by hydrocortisone.
taken as a measure for the inflammatory response. Each experiment was done twice with a minimum of 10 mice.

2.2 HET-CAM assay

The HET-CAM assay was applied using fresh (24 hours), fertile eggs (LOHMANN BROWN hens; average egg weight 64.0-65.0 g, brown egg shells) supplied from a commercial hatchery (R. Schrepper Ltd.; Schottwien/Austria). Defective eggs were discarded. Before incubation the eggs were stored horizontally for 4 hours at room temperature. Then the outer surface of the shell was swabbed with EtOH 70%. The test was performed under sterile conditions. The eggs were put horizontally on trays and rotated at a 2 hours interval during the incubation (incubator: WTB Binder; 72±2 hrs, 37±2°C, relative humidity 80-90%). After incubation the eggs were placed with the large end up. A small hole (1 mm²) was drilled in the egg shell close to the sharp end of the egg. 10 ml of egg white was taken out by a syringe. The suckhole was sealed. On the opposite side the egg shell was opened carefully with a forceps and covered with a parafilm to avoid microbial contamination and desiccation. The eggs were incubated for another 72±2 hours under the conditions mentioned above without rotation. 0.3 ml sodium dodecyl sulphate solution (SDS; 0.5%) were dropped onto exposed CAM as an irritation agent and the start of the irritation phenomena hyperaemia, haemorrhage and lysis were recorded. The test comprised three groups of about 20 eggs each. Pellets of 10 μl each were used. The pellets of the first group consisted only of the carrier agarose (2.5%, 85±2°C) as negative control. The pellets of the second group contained 50 μg SDS in agarose as positive control. Each pellet of the third group was composed of 50 μg SDS and 50 μg suspended test substances in agarose. One pellet was placed on the chorioallantoic membrane of each egg. The eggs were reincubated at 37±2°C for 24 hours. The blood vessels’ irritation phenomena were observed by a binocular magnifier (JVC; magnification 10 fold; Fig. 1).

The findings on the agarose pellet were defined to be the negative control (no inflammation). Inflammation induced by SDS (positive control) was indicated by a typical irritation of the membrane which was characterised by a large number of blood vessels forming a new star-like vascularisation of the granuloma (Fig. 2). This was rated “2”. Medium inflammation (“1”) was rated if only few blood vessels were involved in forming the star-like figure. If the network of blood vessels appeared normal in comparison to the negative control and the granuloma showed no new vascularisation score “0” was rated.

To ensure a neutral evaluation of the visual method all tests were performed by two independent observers.

2.3 Statistical evaluation

A p-value below 0.05 was considered significant for all statistical tests. The anti-inflammatory effect of a substance was defined as the reduction of inflammation on treated subjects relative to positive controls:

\[
\% \text{ reduction} = 100 \times \left(1 - \frac{\text{mean of inflammation of test group}}{\text{mean of inflammation of positive control group}}\right)
\]

In the croton oil test an untreated control group of 20 to 47 mice was made for every dose series of a substance. In the HET-CAM assay a control group of equal size was made for every test. Homogeneity of control groups was tested by analysis of variance for the croton oil test and the Kruskal-Wallis test for the HET-CAM assay. If there was no difference between groups the controls were pooled. 95%-Confidence intervals of anti-inflammatory effects were calculated in the usual way using the formula for the variance of a ratio \(y = x_1 / x_2\) of two random variables (Armitage and Berry, 1987). The null hypothesis that a substance has no anti-inflammatory effect at all was tested using these confidence intervals (Armitage and Berry, 1987). The correlation of egg age and monthly data of egg quality was tested using Spearman correlation coefficients.

3 Results

Eight commercially available constituents were investigated by the croton oil test and the HET-CAM assay in order to ascertain the correlation between the dose administered and the effect observed. The test for heterogeneity of control groups of the HET-CAM assay was not significant (p=0.93). Therefore the
positive control groups were pooled (n=356). The mean inflammation score was 1.68 ±0.052 (mean ± SE). The corresponding test for the positive control groups of the croton oil tests was significant (p<0.0001). In this case the positive controls were not pooled (n=20-47).

The determination of the anti-inflammatory potency of all compounds named above in the two different test systems gave individual trends of inhibition of inflammation (Fig. 3).

The inhibitory effect of rutin depends strongly on the test system applied. In the HET-CAM assay the inhibition observed for a dose of 75 µg was 51.6% in comparison to the less pronounced inhibitory effect of 1.3% at a much higher dose (170 µg) in the croton oil test (IC₅₀ 0.52 µMoles). The dose-response curves do not intersect. Quercetin is more effective than rutin in both test systems. This could be demonstrated clearly by the croton oil test where concentrations of about 170 µg gave inhibitory effects of 79.8% for quercetin and 1.3% for rutin. In this bioassay rutin has an IC₅₀ value of 0.12 µMoles. Only small differences in the effects of the two compounds can be observed by the HET-CAM assay. The two dose-response curves have their point of intersection at a dose of 75 µg with an inhibitory effect of 55.0 ± 4.8%.

In the case of p-coumaric acid, the anti-inflammatory effect is less pronounced in both test systems. The higher sensitivity of the HET-CAM assay can be demonstrated clearly. In this test system the inhibitory effect decreases between 10 µg and 50 µg. The IC₅₀ value obtained for the croton oil test is 1.07 µMoles. The flavonol aglycon apigenin which is similar in structure to quercetin is the most effective substance among all flavonoids tested. The dose-response curves of the two test assays have their point of intersection at a dose of 48.0 µg (inhibition 60.79%). The dose-response curves of acetylsalicylic acid are similar to those of phenylbutazone and indomethacin. They all confirm the high sensitivity of the HET-CAM assay. There is no strong anti-inflammatory effect of the acetylsalicylic acid which can be derived from the IC₅₀ value of the croton oil test (1.36 µMoles). As already observed for p-coumaric acid the dose of 50 µg resulted in decreasing inhibitory effects in comparison to the concentration of 10 µg. The point of intersection for the two curves can be observed at a concentration of 142.5 µg (33.33% inhibition). The stronger anti-inflammatory activity of phenylbutazone and indomethacin compared to acetylsalicylic acid can be proved by both test systems. In the croton oil test the IC₅₀ values were 0.23 for phenylbutazone and 0.26 for indomethacin. The point of inter-
section of the two curves of phenylbutazone were determined for a concentration of 36.5 μg (inhibition 38.15%). Hydrocortisone is the strongest anti-inflammatory agent of these test series. In the HET-CAM assay the maximum effect was demonstrated after administration of 50 μg (91.5% inhibition). With higher concentrations no stronger activity could be achieved. In the croton oil test 0.03 μMoles were estimated as ID₅₀.

After administration of 10 μg the anti-inflammatory substances mentioned in Table 1 caused a stronger effect in the HET-CAM assay than in the croton oil test. The proportion of inhibition is varying to a great extent. The anti-inflammatory effect of the same substance at the same dose differs widely between the two test systems (Tab. 1).

4 Discussion

There is considerable interest in the development of new test methods and applications as alternatives to animal test models because of the animal protection. The HET-CAM assay has been performed as alternative anti-inflammatory test method (Dannhardt et al., 1996) but the comparison with in vivo systems is not clearly defined. The HET-CAM assay is a borderline test between an in vitro and in vivo system where metabolic changes, diffusion and solubility of the substances could be considered. There is a limitation in drawing a direct correlation between in vitro and in vivo studies.

With both test systems the anti-inflammatory effect can be proved. The dose-effect ranges shown in the HET-CAM assay were not corresponding directly to those determined in the croton oil test because of different diffusion, metabolism, and decomposition processes of the substances. The dose-effect correlation between the administered quantity and the inhibition phenomena could be demonstrated mainly for the croton oil test. The Kendall correlation coefficient was used to assess the dose-response relation in the HET-CAM test. Assuming a significance level of 0.05 the dose-response relationship of the tested compounds is not significant. The test results confirm the higher sensitivity of the HET-CAM assay. As there are also different curve courses, it can be concluded that different mechanisms of action are involved.

The results obtained in the HET-CAM assay make clear that although an anti-inflammatory effect could be established for all substances a distinct dose-effect correlation was observed in none of the substances. In the croton oil test pronounced dose-effect curves could be found. The HET-CAM assay is an inexpensive test being easy to manage after a short practical training. This test is appropriate specifically for the routine screening of new compounds or a large number of substances at fixed dose levels in a relatively short space of time. Because of its sensitivity, the HET-CAM assay could be considered a suitable tool for qualitative testing of the anti-inflammatory activity of substances if no appropriate dose-effect curves are required. Other methods have to be applied to investigate precisely the potency of native anti-inflammatory compounds. In the HET-CAM assay much additional information on the toxic or other side-effects of the tested drug may be gained by observing changes in the embryos. The HET-CAM assay as in vitro test model can be applied for investigating the anti-inflammatory effects of herbal drugs, natural and synthetic compounds in the fields of pharmacy as well as chemistry and cosmetics.

References


Tab. 1: Anti-inflammatory effect of substances in varying test systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inhibition (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HET-CAM assay</td>
</tr>
<tr>
<td>Apigenin</td>
<td>55.9 ± 5.9</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>45.4 ± 5.3</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>88.1 ± 5.3</td>
</tr>
</tbody>
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*Concentration of each substance: 10 μg
Verzögerungen auch für die drei ange- 
sprochenen Bereiche der Sicher-
heitsprüfung realisierbar wäre. Die Par-
lamentarier befanden sich jedoch offen-
sichtlich unter Zugzwang, da die 
Kommission bereits signalisierte, dass 
sie konsequente Verbote nicht akzep-
tieren werde. Bei einer konsequenten 
Haltung des Parlaments hätte die 
Erstellung einer neuen Kosmetik-
richtlinie völlig scheitern können, was 
auch aus der Sicht des Tierschutzes 
nicht wünschenswert gewesen wäre. 

Nun wird im Vermittlungsausschuss die 
Kommission zusammen mit dem Parla-
ment anhand der gemachten Vorschläge 
einen Kompromissstext für die Richtlinie 
erarbeiten.

Seit Jahren schon fordert der Deutsche 
Tierschutzbund ein Ende der quä-
lerischen Tierversuche für überflüssige 
Luxusprodukte. Damit spricht er für 87% 
der Bevölkerung in Deutschland, die 
Tierversuche für Kosmetika ablehnen. 

Auch das deutsche Tierschutzgesetz 
verbietet bereits seit 1998 grundsätzlich 
Tierversuche für die Entwicklung von 
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fordert, ist problemlos umzusetzen: "Es 
gibt genügend Kosmetika auf dem 
Markt, bereits getestete Inhaltsstoffe sind 
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genügend Alternativ-Methoden. Schon 
jetzt brauchen die Verbraucher nicht auf 
tierversuchsfreie Kosmetik zu verzichten", 
so die Vizepräsidentin des Deutschen 
Tierschutzbundes, Dr. Brigitte Rusche. 

Bereits vor über 20 Jahren hat der 
Deutsche Tierschutzbund Richtlinien für 
tierversuchsfreie Kosmetika entwickelt. 

Zwar sollen Tierversuche innerhalb der 
EU nach Willen des EU-Parlamentes für 
Kosmetika ab 2005 endgültig verboten 
werden. Doch konnte man sich nicht zu 
dem ebenfalls dringend erforderlichen 
konsequenten Vermarktungsverbot für 
tierexperimentell getestete Kosmetika 
nach fünf Jahren durchringen. Noch 10 
Jahre lang sollen Ausnahmen hiervon 
zugelassen werden. Allerdings sind die 
Ausnahmen wesentlich eingeschränkter, 
also die Kommission und der Ministerrat 
vorgeschlagen haben.

"Diese Entscheidung ist für uns sowie 
die Millionen deutschen Bürger zwar 
eine Enttäuschung, aber es ist weitaus 
mehr, als was die Kommission und 
der Ministerrat vorgeschlagen haben", 
erklärt Wolfgang Apel, Präsident des 
Deutschen Tierschutzbundes. "Wir 
müssen uns nunmehr dafür einsetzen, 
dass die Kommission im Vermittlungs-
ausschuss nicht noch weitere Ausnahmen 
und zeitliche Verzögerungen durchsetzt."

Das Europäische Parlament hat am 
11. Juni 2002 im Rahmen der zweiten 
Lesung zur 7. Änderung der EU-
Kosmetikrichtlinie zwar einem end-
gültigen Tierversuchsverbot für 
Kosmetika innerhalb der EU ab 2005 
zugestimmt, jedoch ein konsequentes 
Vermarktungsverbot abgelehnt. In drei 
von ca. 12 Bereichen der Sicherheits-
prüfung von Chemikalien soll erst nach 
zehn anstatt nach fünf Jahren nach 
Annahme der Richtlinie ein Ver-
marktungsverbot in Kraft treten. Ur-
sprünglich wollte das Parlament ein 
endgültiges Aus nach fünf Jahren. Aus 
pluraler Sicht der Deutschen Tierschutz-
bundes (DTB) wäre dies bereits ein 
 großzügiges Zugeständnis an die Indust-
rie für ein Verbot gewesen, das bereits 
1993 versprochen wurde und ohne weit-
ere Verzögerungen auch für die drei 
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aus einer Pressemeldung des DTB vom 
11.6.2002