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.

Zusammenfassung: Der HET-CAM Assay als *in vitro* Alternative zum Crotonöl-Test zur Untersuchung der antiinflammatorischen Wirkung von steroiden und nicht-steroiden Verbindungen

In dieser Studie wurden die Irritationsphänomene an der Chorionallantoismembran (CAM) des bebrüteten Hühnereis als in vitro Modell (HET-CAM Test) mit dem Crotonöl-Test als in vivo Methode verglichen. Die Untersuchungen der antiinflammatorischen Aktivität wurden mit folgenden steroiden und nicht-steroiden Substanzen durchgeführt: Hydrocortison, Indomethacin, Phenylbutazon, Acetylsalicylsäure, Rutin, Quercetin, Apigenin und p-Cumarsäure. Zum ersten Mal wurden die zwei Methoden miteinander verglichen und bewertet mit dem Ziel, Tierversuche zu reduzieren. Eine mögliche in vitro–in vivo Korrelation sollte untersucht werden, ebenso die Möglichkeit, das Tiermodell durch einen in vitro Test zu ersetzen.

In beiden Testmodellen konnte für die untersuchten Verbindungen eindeutig antiinflammatorische Aktivität nachgewiesen werden, die allerdings unterschiedliche Tendenzen zeigte. Der in vitro HET-CAM Test wies eine größere Empfindlichkeit auf als der in vivo Crotonöl-Test, in dem die untersuchten Substanzen eindeutige Dosis-Wirkungs-Korrelationen zeigten. Im modifizierten HET-CAM Assay konnte keine eindeutige Dosis-Wirkungs-Beziehung nachgewiesen werden. Dieses Testsystem ist auf Grund seiner Empfindlichkeit für eine qualitative Testung der antiinflammatorischen Aktivität von Substanzen sehr gut geeignet, sofern keine genauen Dosis-Wirkungskurven benötigt werden. Außerdem ist der preisgünstige Test innerhalb kurzer Zeit zu erlernen. Aus den vorliegenden Ergebnissen kann abgeleitet werden, dass die unterschiedlichen Dosis-Wirkungs-Kurven möglicherweise auf verschiedene Wirkmechanismen zurückzuführen sind.

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 multimediated 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 vitro-in vivo* 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 embryo toxicity (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 inhibition 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.

Test period: April 1999 – March 2000.

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 (1cm^2) 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

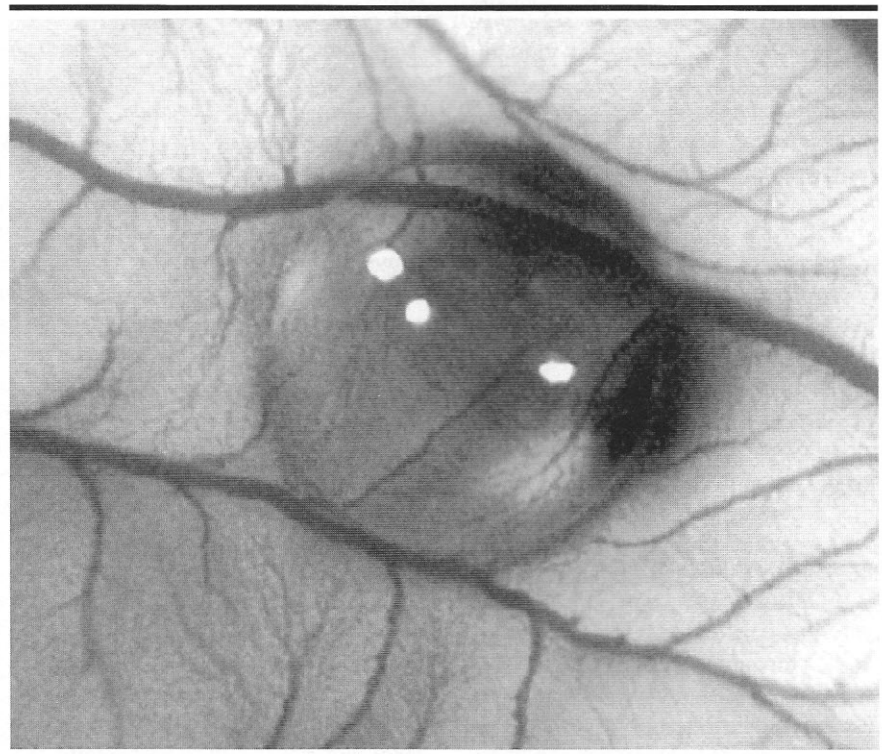

Fig. 1: HET-CAM assay. Inhibition of the inflammation of the hen's egg chorioallantoic membrane (CAM) by hydrocortisone.

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 batchery (R. Schropper 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 mm^2) 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

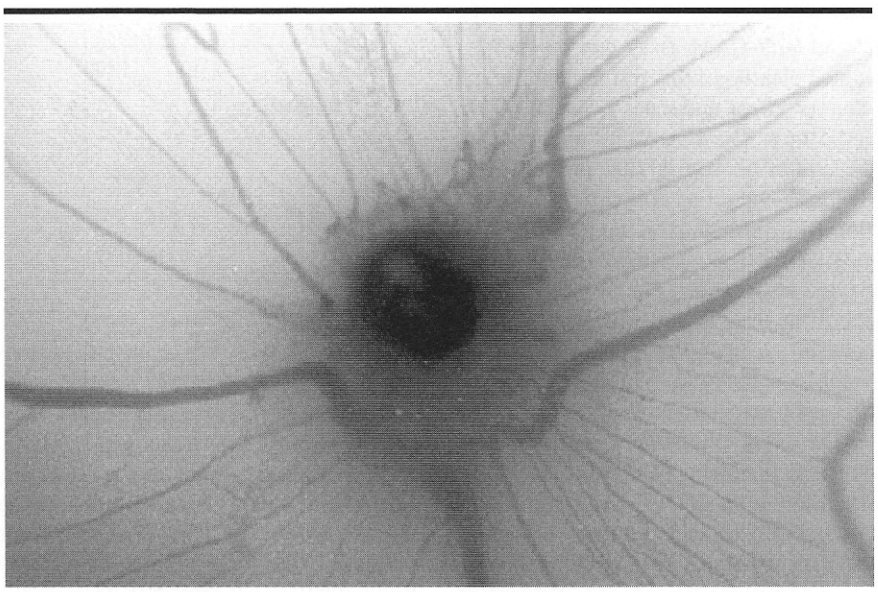

Fig. 2: HET-CAM assay. Inflammation of the CAM irritated by sodium dodecyl sulphate (SDS).

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 (ID_{50} 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 ID_{50} 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 IC_{50} 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 IC_{50} 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 IC_{50} values were 0.23 for phenylbutazone and 0.26 for indomethacin. The point of inter-

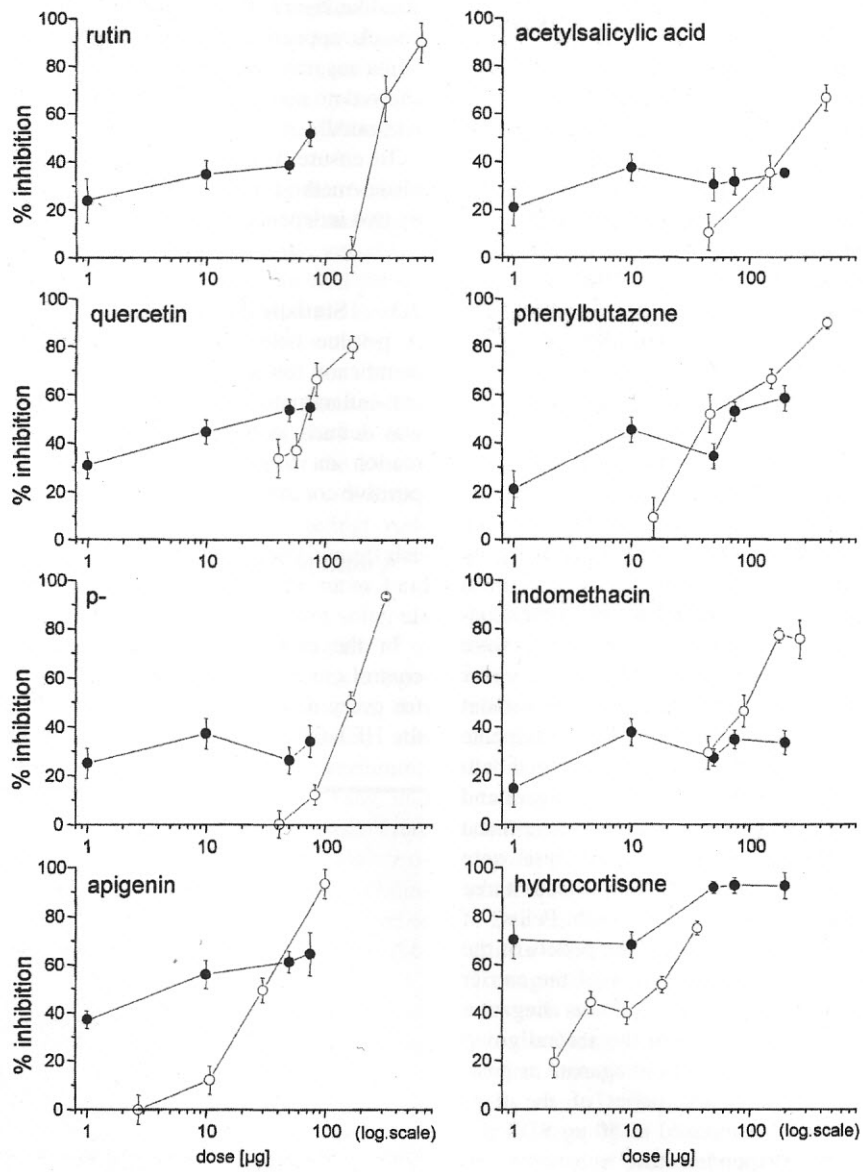

Fig. 3: Inhibition of inflammation stimulated by croton oil on mouse ears (◦: Croton oil test, animals treated with test compounds: n = 41-83, animals for positive control: n = 20-47). Inhibition of inflammation stimulated by SDS on the CAM of chicken embryos (•: HET-CAM assay, eggs treated with test compounds: n = 60-180, eggs for positive control: n = 356). Dose-response-curves of anti-inflammatories (rutin, quercetin, p-coumaric acid, apigenin, acetylsalicylic acid, phenylbutazone, indomethacin, hydrocortisone). Results + / - SE given as percentages of means of experiments relative to positive controls.

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 ID_{50}.

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.

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

Substance*	Inhibition (% ± SEM)			
	HET-CAM assay	Croton oil test	ID_{50} (µ Moles)	Proportion
Apigenin	55.9 ± 5.9	11.1 ± 5.8	0.10	5.0 : 1
Phenylbutazone	45.4 ± 5.3	5.9 ± 8.4	0.23	7.7 : 1
Hydrocortisone	68.1 ± 5.3	43.6 ± 4.7	0.03	1.5 : 1

*Concentration of each substance: 10 µg

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Sondermeldungen aus aktuellem Anlass:

EU: Kein endgültiges Aus für Tierversuche in der Kosmetik in diesem Jahrzehnt

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, als 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 Vermittlungsausschuss 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 endgültigen Tierversuchsverbot für Kosmetika innerhalb der EU ab 2005 zugestimmt, jedoch ein konsequentes Vermarktungsverbot abgelehnt. In drei von ca. 12 Bereichen der Sicherheitsprüfung von Chemikalien soll erst nach zehn anstatt nach fünf Jahren nach Annahme der Richtlinie ein Vermarktungsverbot in Kraft treten. Ursprünglich wollte das Parlament ein endgültiges Aus nach fünf Jahren. Aus der Sicht des Deutschen Tierschutzbundes (DTB) wäre dies bereits ein großzügiges Zugeständnis an die Industrie für ein Verbot gewesen, das bereits 1993 versprochen wurde und ohne weitere Verzögerungen auch für die drei angesprochenen Bereiche der Sicherheitsprüfung realisierbar wäre. Die Parlamentarier befanden sich jedoch offensichtlich unter Zugzwang, da die Kommission bereits signalisierte, dass sie konsequente Verbote nicht akzeptieren werde. Bei einer konsequenten Haltung des Parlamentes hätte die Erstellung einer neuen Kosmetikrichtlinie völlig scheitern können, was auch aus der Sicht des Tierschutzes nicht wünschenswert gewesen wäre. Nun wird im Vermittlungsausschuss die Kommission zusammen mit dem Parlament anhand der gemachten Vorschläge einen Kompromisstext für die Richtlinie erarbeiten.

Seit Jahren schon fordert der Deutsche Tierschutzbund ein Ende der quälerischen 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 Kosmetika. Das sofortige und vollständige Vermarktungs- und Tierversuchsverbot, wie es der Deutsche Tierschutzbund fordert, ist problemlos umzusetzen: „Es gibt genügend Kosmetika auf dem Markt, bereits getestete Inhaltsstoffe sind ausreichend vorhanden und es existieren 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.

aus einer Pressemitteilung des DTB vom 11.6.2002