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Article

Corrositex®, BCOP and HET-CAM as alternative methods to animal experimentation

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Tests in animals are used as models in toxicological and investigative studies. However, such tests have been considered inhumane because they can cause pain and suffering to experimental animals, while these methods can often be subjective. Protests calling for animal protection have questioned the effectiveness of *in vivo* tests and suggest the introduction of alternative, *in vitro* methods. International organizations, such as the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Institute of Health (NIH), the Organization for Economic Co-operation and Development (OECD), that regulate and develop new alternative animal models, have indicated the running of preliminary assays and execution of sequential tests, which consider physical-chemical properties and data of *in vitro* assays, before performing *in vivo* studies. Towards this background, the objective of the present article was to select promising alternative methods such as Corrositex®, BCOP and HET-CAM, intended to refine or replace the use of animals and reduce their suffering.

Uniterms: Animal experimentation/alternative methods *in vitro*. Experiments/*in vitro* methods. Corrositex®/*in vitro* method/experiments. BCOP/*in vitro* method/experiments. HET-CAM/*in vitro* method/experiments.

Testes em animais são utilizados como modelos em estudos toxicológicos e de pesquisa. Entretanto, tais testes têm sido considerados desumanos, porque causam dor e sofrimento aos animais experimentais, porquanto estes métodos podem, freqüentemente, ser subjetivos. Protestos clamando pela proteção animal têm questionado a eficácia dos testes *in vivo* e sugerem a introdução de métodos alternativos *in vitro*. Organizações internacionais, tais como Comitê de Coordenação Interagências de Métodos de Validação Alternativos (ICCVAM), Instituto Nacional de Saúde (NIH), Organização para Cooperação Econômica e Desenvolvimento (OECD), que regulam e desenvolvem novos métodos alternativos aos modelos animais, indicaram a realização de ensaios preliminares e a execução de testes seqüenciais, que consideram as propriedades físico-químicas e os dados dos ensaios *in vitro*, antes de efetuarem estudos *in vivo*. Nessa direção, o objetivo do presente artigo foi selecionar métodos alternativos promissores, tais como Corrositex®, BCOP e HET-CAM, com o intuito de aperfeiçoar ou substituir o uso de animais e reduzir seus sofrimento.

Unitermos: Animais experimentais/uso/métodos substitutos *in vitro*. Experimentos/métodos *in vitro*. Corrositex®/método *in vitro*/experimentos. BCOP/método *in vitro*/experimentos. HET-CAM/método *in vitro*/experimentos.

INTRODUCTION

For many decades, movements for the protection of animals have called the effectiveness of *in vivo* tests into question, and suggested introducing the alternative *in vitro* and *ex vivo* methods. Extensive research in the scientific community has been encouraged to reduce and replace the use of laboratory animals in biological experiments (Marona, Lucchesi, 2003; Chorilli, Michelin, Salgado, 2007). However, use of experimental animals cannot yet

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be totally abandoned in trials assessing the safety of many products (CCAC, 1993; FELAS, 1999; CPCSEA, 2003; Marona, 2003; Marona, Lucchesi, 2004).

In using *in vivo* biological material, it is important to ensure physical integrity, while taking into account microbiological contamination, genetics, nutrition and proper handling, in order to avoid invalid conclusions in the experiments or unnecessarily increasing the number of animals tested (Politi, Salgado, Pietro, 2008). It is important to stimulate research to allow the replacement of living biological material by cultures of cells or other objects of study, and to obtain valid and reliable techniques that allow the use of pharmaceuticals and cosmetics.

For a method to be accepted, it must undergo assessment to establish its reliability and relevance. To achieve this, it is necessary to harmonize the processes of validation through International Committees, such as the ICCVAM - Interagency Coordinating Committee on the Validation of Alternative Methods, which is composed of 15 regulatory and research agencies including the Environmental Protection Agency (EPA) and Food and Drug Administration (FDA). The ICCVAM, through its agencies, discusses the development, validation, acceptance and national and international harmonization of toxicological tests through the Federal government of the United States (Cazarini, Corrêa, Zambrone, 2004).

International ethical standards recommend the judicious use of laboratory animals and development and use of alternative test methods. In Brazil, the first attempt to regulate the ethical use of animals was through law nº 6638, 1979, which sets standards for teaching and scientific practice of vivisection of animals. In 1998, law nº 9605 stated that "cruel or painful experiments on live animals, even for educational or scientific purposes, where there are alternative resources" is classified as a crime. In 2008, law nº 11794, known as the Arouca Law, a legal benchmark in Brazil for the scientific use of laboratory animals, encouraged the substitution of animals by alternative methods (Brasil, 1979, 1998, 2008a, 2008b).

The evaluation of potential skin irritants through the use of alternative methods has been the subject of major discussion. To reduce suffering and the number of animals used, the running of preliminary testing and implementation of sequential tests have been suggested, i.e. from the most simple to sophisticated testing, by considering the physical and chemical properties and *in vitro* test results, before conducting studies in vivo (Cazarini, Corrêa, Zambrone, 2004).

In order to explore the use of alternative methods, this work sought to provide a brief presentation of three *in vivo/ex vivo* methods, namely: the dermal corrosion test (Corrositex®), the Bovine Corneal Opacity and Permeability (BCOP) test, and the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM).

Dermal corrosion test method

Corrositex® is an *in vitro* test method developed by In Vitro International, proposed as an *in vitro* alternative test method to *in vivo* methods for assessing the potential of chemicals to cause skin corrosion (IN VITRO, 2008). It was officially approved by the ICCVAM at a public meeting held on the 21st of January, 1999 in Bethesda (MD), U.S.A. This entailed a detailed review of studies that analyzed the efficacy and limitations of the method to identify potential corrosive chemicals, and a final report which resulted in a document called the Peer Review Panel (PRP). This report was organized into seven topics, as follows: (1) Test Method Description; (2) Test Method Data Quality; (3) Test Method Performance; (4) Test Method Reliability (Repeatability/Reproducibility); (5) Other Scientific Reviews; (6) Other Considerations; and (7) Related Issues. Each item was reviewed by two or five PRP committee members (NIH, 1999).

Corrositex® was approved by the PRP for the testing of acids, bases and acid derivates by the DOT (Department of Transportation of United States). After one year, Corrositex® was accepted by other American agencies including the Consumer Product Safety Commission (CPSC), the Environmental Protection Agency (EPA), the Food & Drug Administration (FDA), the International Air Transport Association (IATA), the National Institute of Environmental Health Sciences (NIEHS), the Occupational Safety and Health Administration (OSHA) and Transport Canada. In 2006, Corrositex® was published by the European OECD (Organization for Economic Co-operation and Development) as TG 435 (NTP, 2008).

The final PRP report presented the method technologies along with their advantages and disadvantages, topics which shall be addressed below. Corrositex® assesses whether a substance can produce skin corrosion and classifies substances according to the UN Packing Groups of the US – DOT. The UN Packing Groups system classifies substances according to their danger in transportation and storage processes. Materials corrosive to skin belong to packing group Class 8. In this class, substances are separated into three subgroups: (I) great danger, (II) medium danger and (III) least danger (SAMPLE, 2003).

The grading method is based on corrosion of a membrane or coloration of a detection system. This model mimics the *in vivo* effect of corrosive substances on skin, where material used consists of a plastic tube filled

with a chemical detection system (CDS), covered with a membrane.

The test chemical is placed on the membrane. Corrosive substances penetrate the membrane and produce a color change in the CDS. For non-corrosive substances, the membrane stays intact. The time it takes for these changes to occur can be observed by an operator using a simple chronometer (NIH, 1999).

Preparation of the membrane matrix must be done at least two hours prior to performing the assay. The test kit comes with powder membrane matrix and its diluent. To carry out the procedure, this must be stirred and warmed to 70 ºC in a water bath until the membrane matrix has completely dissolved. The preparation should then be refrigerated (between 2 ºC and 8 ºC).

Before starting the assay, it is possible to verify whether the sample is adequate to be tested by Corrositex®. This pre-analysis is called the qualification test. The kit provides an indicator known as the qualification tube, which may change in color when the sample is added. If the qualification test yields a positive result, then the process can proceed to the categorization stage. In this stage, the color change obtained after introducing the sample into the CDS tube is compared to the corresponding color chart (Figure 1). Samples are separated into categories 1 and 2, according to the color change (Table I).

The next step is to classify the corrosive agent according to the UN Packing Groups I, II and III. The sample is placed on the membrane that covers the CDS tube. If the tested substance corrodes the membrane, then the corrosion time is recorded (Figure 2).

According to the recorded time and category that the substance was assigned into during the categorization stage, the substance is finally classified as UN Packing Group I, II or III (Table I).

Corrositex® has the main advantage of preventing animal suffering and being fast to execute, since the test duration ranges from three minutes to four hours. Moreo-

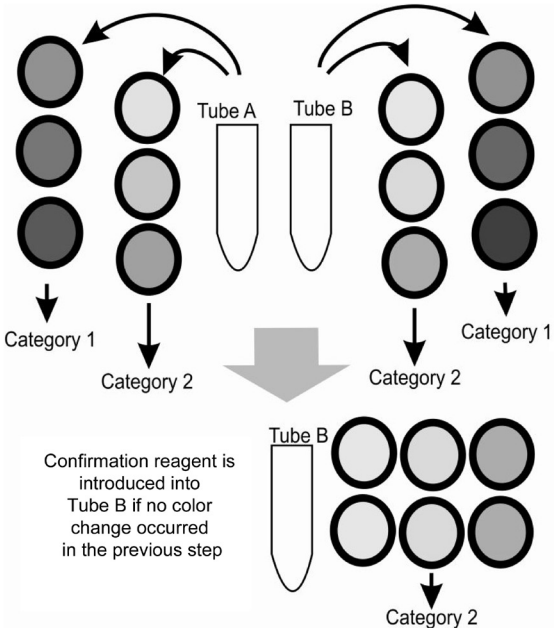

FIGURE 1 – Categorization stage. The kit contains Tubes A and B. The sample is introduced into both tubes and shaken. If a color change is observed in either tube, this color is matched to the corresponding color chart and assigned the appropriate category. If a color change is not observed in either tube, add two drops of the confirmation reagent to Tube B. Match the resulting color to the color charts, assigning the category 1 or 2 as appropriate (*In Vitro* International, 2008).

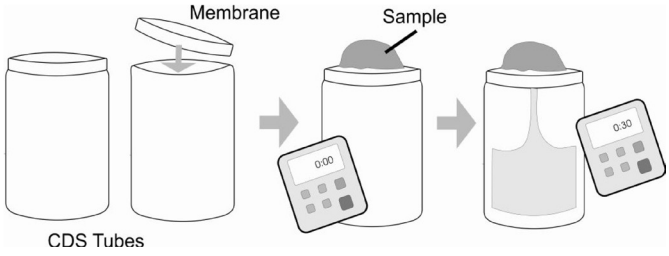

FIGURE 2 – Classification stage. Sample is classified according to UN Packing Groups I, II or III. Sample is placed on a membrane which covers the CDS tube. Time taken to corrode the membrane is recorded. Based on the category that the agent was assigned previously and the time taken to corrode the membrane, the samples are classified into Groups I, II or III (*In Vitro* International).

TABLE I - UN Packing Group assignment Table. Group I: great danger; Group II: medium danger; Group III: least danger (DOT)

Category 1			Category 2		
Corrosivity	Packing group	Mean time	Corrosivity	Packing group	Mean time
Corrosive	I	0 to 3 min	Corrosive	I	0 to 3 min
Corrosive	II	> 3 min to 1 h	Corrosive	II	> 3 to 30 min
Corrosive	III	> 1 to 4 h	Corrosive	III	> 30 to 60 min
Non-Corrosive	Not applicable	> 4 h	Non-Corrosive	Not applicable	> 60 min

Table adapted from NIH, 1999.

ver, it is more cost-effective than dermal corrosion testing in rabbits. Only a small work area is necessary and a single operator can carry out ten tests per day. Comparing to the pH physical-chemical test, Corrositex® is more accurate in detecting false-positives; however, for extremities of pH, both the tests are matched in performance. The disadvantage of the test is that it can be indicated for only some chemical substances such as acids, acid derivatives, acyl halides, alkylamines and polyalkylamines, bases, chlorosilanes, metalhalides, and oxyhalides (NIH, 1999).

Bovine Corneal Opacity and Permeability (BCOP) Assay

Bovine corneas have been used since the 1940s as an alternative model of the human eye, in conjunction with the BCOP (Bovine Corneal Opacity and Permeability Assay) a useful parallel for possible human exposure. BCOP assessments were developed by Pierre Gautheron and Dukic (1992) as an alternative to the Draize test. The purpose of the BCOP test is to evaluate the potential corrosion/irritation of an eye from test material as a measure of the capacity of inducing opacity and/or corneal permeability. Corneal opacity may be caused by protein denaturation, precipitation, or the induction of cellular swelling of the stroma. Corneal permeability, assessed by fluorescein, is characterized by a loss of junctions and cell membrane barrier properties of the corneal epithelium (Cazarin; Corrêa; Zambrone, 2004; NIEHS, 2006).

The test procedures are presented in Figure 3. Briefly, bovine eyes were collected soon after the death of the animal and placed in cool saline solution. The corneas are carefully examined in order to select those that have no type of injury, and these are placed in the special BCOP chamber together with the minimum essential medium (MEM) which remains in contact with the epithelium and endothelium of the cornea. After incubation for 1 hour at 32° C, an initial reading of the opacity (pre-test) is performed using an opacitometer.

The corneas were divided into 2 groups: samples and controls (groups of 3 to 6 corneas by objective test). Positive and negative controls are the basic criteria for test acceptance and reproducibility. Individual positive controls are used to test liquid and solid materials using 100% ethanol for the liquid protocol and 20% imidazole in MEM for the solid protocol.

The cornea is exposed to the material to be tested, which may be at any concentration in a variety of solvents, and involve a wide range of time intervals for different potential eye irritants: from the severe to corrosive. The test material is next washed and the cornea is incubated again (additional incubation). The time of incubation for the demonstration of additional signs of irritation can vary. After this period, the opacity is read and recorded for each cornea. The values of the opacities obtained by additional incubation are subtracted from the opacity values read in the pre-test in order to give the correct value (NIEHS, 2006).

The permeability test is performed by measuring the passage of fluorescein marker through the cornea. An average of the sample is taken from the posterior chamber and measured spectrophotometrically (490 nm) to determine the amount of fluorescein available. Different values of optical densities are measured by the amount of fluorescein that permeates through the cornea to accumulate in the posterior chamber with MEM. The intensity of the response increases with the amount of fluorescein across the cornea, and the values for classification of irritants are depicted in Table II. The calculation is carried out according to the following equation:

In Vitro Score = opacity value + 15 × OD490 value

TABLE II - In vitro values for classification of irritants

In Vitro Score	Results
0 to 25	Mild irritant
25.1 to 55	Moderate irritant
≥ 55.1	Severe irritant

The cornea can also be fixed, sectioned and examined histologically (by a group of pathologists) (Cazarin, Corrêa, Zambrone, 2004; NIEHS, 2006).

Figure 3 shows a schematic representation of the BCOP test.

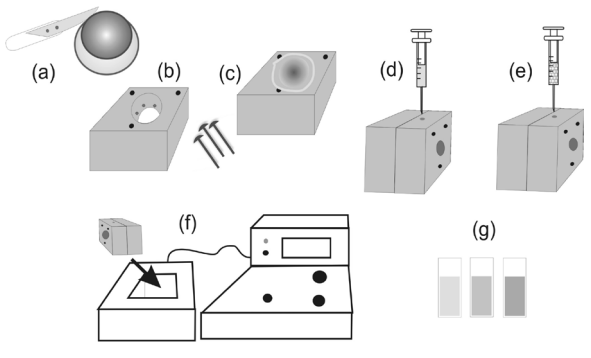

FIGURE 3 - (a) bovine eyes were collected soon after death of the animal (b) special BCOP chamber (c) the corneas were placed in a special BCOP chamber and MEM added (d) the cornea is exposed to the material under test (e) control (f) the opacity is measured for each cornea (g) permeability test. Figure adapted from CAMVA, 2008.

The BCOP presents some limitations including: (I) it cannot assess late response;(II) it does not have tear film, which increases the chance of false positives, (III) there must be a slaughterhouse near the laboratory; and (IV) the test system is devoid of blood flow and nerve tests, which preclude observation of inflammatory effects of the iris. However, the BCOP offers several advantages: (I) reduction of test time and (II) low cost; (III) provides histological evaluation of the corneal tissue; and (IV) the permeability test can be quantified (Cazarin, Corrêa, Zambrone, 2004; NIEHS, 2006). The BCOP is the first scientifically-validated alternative method to gain acceptance as a test for eye safety by the U.S.A. regulatory agencies making up the ICCVAM (NEWLY, 2008).

Hen´s Egg Test on the Chorioallantoic Membrane (HET-CAM)

The *Hen's Egg Test on the ChorioAllantoic Membrane* (HET-CAM) is another alternative method to animal experimentation for assaying corrosives and/or severe ocular irritants, using chorioallantoic membrane of embryonated hen's egg. This test assesses the damage to this membrane to determine the potential irritation to the conjunctiva. The acute effects of the test substance on the small vessels and proteins of the soft tissue of the membrane are assumed to be similar to the effects caused by the substances in the eyes of rabbits (CAMVA, 2008).

The HET-CAM was described by Luepke in 1985 to assay irritant/corrosive potential and allows the study of the immediate effects of administration of the test substance on solids or liquids in membrane of embryonated hen's egg. This method is internationally validated (Silva, Rougier, Dossou, 1992; Cazarin; Corrêa; Zambrone, 2004).

The chorioallantoic membrane (CAM) is a complete tissue containing arteries, veins and capillaries and is technically easy to study. The CAM responds to injury with an inflammatory process similar to that observed in the conjunctival tissue of the eyes of rabbits (Draize test). Its well-developed vascularization provides an ideal model for studies of ocular irritation.

The principle of the method is based on the test substance (solid, 0.3 g and liquid, 0.1 to 0.3 mL) in chorioallantoic membrane of fertilized eggs (9 to 10 days of development) and the results are assessed by type of irritation (lysis, bleeding or coagulation) (Cazarin; Corrêa; Zambrone, 2004).

The procedure of the HET-CAM test includes five phases: (I) preparation of the egg, (II) preparation of sample and positive control, (III) procedure of assay, (IV) scoring phase of eggs and (V) calculation of the score for irritation. In phase (I) the fertilized hen's eggs (day 0), received and analyzed for the presence of the damage, are cleaned with 70% alcohol and are placed in an incubator at controlled temperature and humidity (37 ± 1 °C). On the fourth day, the eggs are viewed under light to verify the existence and position of the embryo, and then egg shells are opened, with each membrane carefully dampened with 2-3 mL of 0.9% saline. A quantity of 3.0 mL of albumin is removed from the eggs. The shell opening is then sealed with a transparent tape and the eggs returned to the incubator. The viability of the eggs is verified every working day. On the tenth day, a ring of Teflon is placed in the CAM of each egg that serves as a reservoir to dilute the test samples.

In phase (II), the samples are suspended in water or vegetable oil or another appropriate solvent. Sodium lauryl sulfate (SLS) is used as a positive control, diluted with water to 0.01*M*. Ten eggs are kept closed and observed daily for viability, serving as a control group to monitor environmental conditions.

In phase (III), on the tenth day the eggs are removed from the incubator and 40 µL of test sample is diluted. The (SLS) or solvent is added to the ring within each egg in a group of 8 to 10 eggs. The eggs are placed back in the incubator for 30 minutes ± 5 minutes. The area inside the ring is evaluated for vascular damage with a flashlight, using the area outside the ring as a guide. This damage might be lysis, bleeding and / or coagulation. The eggs are evaluated for the severity of reactions at 1 and 5 minutes. All the effects observed in each egg for each group are recorded. If any vascular effect is observed, the results are considered positive. According to the positive or negative response, a value of RC_{50} (the concentration of test product resulting in 50% of eggs showing positive response) will be determined.

In phase (IV), the appropriate eggs available are removed from the incubator and the tapes taken off. The eggs were shelled to increase the visible area of chorioallantoic membrane. The vascular effects are classified according to the criteria described in Table III. In phase (V), irritation score are calculated using the following equation (Cazarin; Corrêa; Zambrone, 2004; CAMVA, 2008):

$$\left(\left(\frac{(301 - Hemorrhage\ Time)}{300}\right)\times 5\right)+\left(\left(\frac{(301 - Lysis Time)}{300}\right)\times 7\right)+$$

$$+\left(\left(\frac{(301 - Coagulation Time)}{300}\right)\times 9\right)$$

TABLE III - *Scores* comparing the vascular effects with the description of observation

Vascular effects	*Scores*	Description of comment
0	0	No reactions observed, CAM normal
Ghost vessels	1	Vessels devoid of blood flow, appearing clear
Capillary injection	2	Hyperemia or increased blood flow in small blood vessels of the CAM
Minimal bleeding	3	Bleeding potential covering no more than 25% of the area of the ring
Mild bleeding	4	Drops of blood covering 25 – 50% of the area of the ring
Moderate bleeding	5	Drops of blood covering 50 – 75% of the area of the ring
Severe bleeding	6	>75% of the area inside the ring is covered with blood pools, dark areas might be formed as shells

Table adapted from CAMVA, 2008.

where:
Hemorrhage Time = time (in seconds) of the first appearance of blood hemorrhages
Lysis Time = time (in seconds) of the first appearance of vessel lysis
Coagulation Time = time (in seconds) of the first appearance of protein coagulation

The relationship between scores and category of irritation is found in Table IV.

TABLE IV - Relationship of scores with category of irritation

Scores on HET-CAM	Category of irritation
0 – 0.9	Nonirritant or practically no irritation
1 – 4.9	Weak or slight irritation
5 – 8.9 or 5 – 9.9	Moderate irritation
9 – 21 or 10 - 21	Strong or severe irritation

Table adapted from CAMVA, 2008.

The advantages of this method include reduced time and cost of testing while quantitative analysis employed in some parameters. Limitations include the impossibility of assessing late responses, absence of tear film, which increases the chance of false positives, and inability to assess reversible damage (Cazarin, Corrêa, Zambrone, 2004; CAMVA, 2008).

Figure 4 shows the schema of the HET-CAM test.

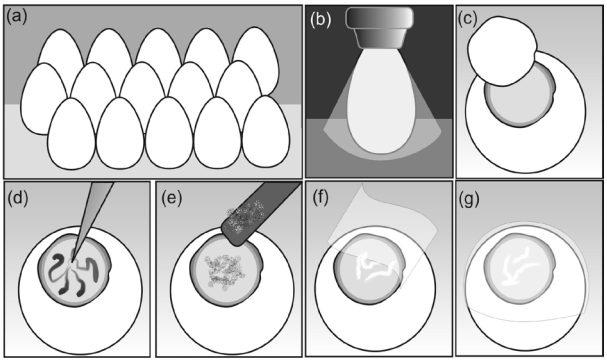

FIGURE 4 - Schema of the procedure of the HET-CAM test, in which (a) indicates receipt of eggs, their verification and storage in incubator; (b) examination of the eggs on fourth day to verify the existence and position of embryo; (c) opening of the shell eggs and wetting; (d) addition of test substance liquid; (e) addition of test substance solid; (f) closure of the opening with transparent tape; (g) assessment within of the ring for vascular damage. Figure adapted from CAMVA, 2008.

DISCUSSION

The method of evaluation in stages is appropriate for the general assessment of the toxicity of a substance and can represent a useful tool to achieve the objectives of the 3Rs program (Refine, Reduce, Replacement), because it can improve the process of identifying hazards and reduce the time and costs of this evaluation (Cazarin, Corrêa, Zambrone, 2004).

The Corrositex® showed excellent values of sensitivity, specificity and accuracy for the substances and chemical mixtures analyzed (NIH, 1999). Therefore, even though it may not be able to completely replace the use of animals, the test is appropriate to refine and reduce experimental animal use.

Alternative methods for assessing ocular irritation could be used in combination for additional determination of the classification of ocular irritation. There are four internationally validated methods: BCOP, HET-CM, ICE (Isolated Chicken Eye) and IRE (Isolated Rabbit Eye), but only the first two have been validated for use in Brazil. These methods identify severe irritants and corrosive substances and were recommended by the US-EPA as pre-tests for assessment of irritant/corrosive potential. However, at present, none of the four protocols can fully replace *in vivo* testing in rabbit eyes. The perspectives for these methods include evolution of BCOP test for qualifying mild and moderate ocular irritants, and improvement of the HET-

CAM method for assessment of severe ocular irritants and corrosive substances, allowing its use for identification of mild and moderate irritants.

Despite the potential of alternative methods presented in this study, they are not widely used and few studies in the literature have employed these methods. However, these tests should be given merit because they offer objectivity and standardization of results. in contrast to the methods involving animals which produce subjective results.

CONCLUSION

The methods of dermal corrosion, BCOP and HET-CAM are valid and can be reliably applied in research on potentially toxic substances. Although these alternative methods still have limitations and are unable to fully replace the use of animals, they can contribute toward refining and reducing animal experiments and also decrease animal suffering.

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REFERENCES

BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP). Available at: <http://www.iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_brd_bcop.htm>. Accessed on: 29 jul. 2008.

BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP). ASSAY. Available at: <http://www.iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_brd_bcop.htm>. Accessed on: 29 jul. 2008.

BRASIL. Agência Nacional de Vigilância Sanitária. Legislação. VisaLegis. *Lei n.9.605, de 12 de fevereiro de 1998*. Dispõe sobre as sanções penais e administrativas derivadas de condutas e atividades lesivas ao meio ambiente, e da outras providencias. Available at: <http://e-legis.anvisa.gov.br/leisref/public/showAct.php?id=455&word=>. Acessed on: 23 mar. 2009.

BRASIL. Agência Nacional de Vigilância Sanitária. *Guia de avaliação de segurança de produtos cosméticos*. Available at: <http://www.anvisa.gov.br/cosmeticos/guia/html/pag05.htm>. Accessed on: 10 jul. 2008.

BRASIL. Presidência da República. Lei n.6.638, de 8 de maio de 1979. Estabelece normas para a prática didático-científica da vivissecção de animais e determina outras providências. Available at: <http://www.planalto.gov.br/ccivil_03/LEIS/1970-1979/L6638.htm>. Acessed on: 23 mar. 2009.

BRASIL. Presidência da República. Lei n.11.794, de 8 de outubro de 2008. Estabelece procedimento para o uso científico de animais. Available at: <http://www.planalto.gov.br/ccivil_03/_ato2007-2010/2008/lei/l11794.htm>. Acessed on: 22 nov. 2008.

CANADIAN COUNCIL ON ANIMAL CARE. Available at: <http://www.iivs.org/pages/methods/CAMVAsummarysheet.pdf>. Accessed on: jul. 2008.

CAZARIN, K. C. C.; CORRÊA, C. L.; ZAMBRONE, F. A. D. Redução, refinamento e substituição do uso de animais em estudos toxicológicos: uma abordagem atual. *Rev. Bras. Ciênc. Farm.*, v.40, n.3, p.289-299, 2004.

CANADIAN COUNCIL ON ANIMAL CARE. *Guide to the care and animal use of experimental*. 2.ed. Otawa: Canadian Council on Animal Care, 1993. v.1, 212 p.

CHORILLI, M.; MICHELIN, D. C.; SALGADO, H. R. N. Animais de laboratório: o camundongo. *Rev. Ciênc. Farm. Básica Apl.*, v.28, n.1, p.11-23, 2007.

COMMITTEE FOR THE PURPOSE OF CONTROL AND SUPERVISION ON EXPERIMENTS ON ANIMALS. CPCSEA Guidelines for laboratory animal facility. *Indian J. Pharmacol.*, v.35, n.4, 257-274, 2003.

CUELLAR, N.; LLOYD, P. H. Phase two: evaluating the eye irritancy of solventes in a single fragrance mixture with the bovine corneal opacitu and permeability (BCOP) assay. *Toxicologist*, v.78, n.1, p.268-284, 2004.

FEDERATION OF EUROPEAN LABORATORY ANIMAL SCIENCE ASSOCIATIONS. FELASA guidance paper for the accreditation of laboratory animal diagnostic laboratories. *Lab. Anim.*, v.33, suppl.1, p.S19-S38, 1999.

GAUTHERON, P.; DUKIC, M.; ALIX, D. SINA, J. F. Bovine corneal opacity and permeability test: an *in vitro* assay of ocular irritancy. *Fund. Appl. Toxicol.*, v.18, p.442-449, 1992.

ICCVAM. *ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods*. Research Triangle Park: National Toxicology Program, 2003. p.5-7 (NIH Publication n.03-4508).

IN VITRO INTERNATIONAL. Available at: <http://www.invitrointl.com>. Accessed on: 24 feb. 2008.

INSTITUTE FOR IN VITRO SCIENCES. "Validation Update". Available at: <http://www.iivs.org/pages/valupdate.php>. Accessed on: 10 jul. 2008.

MARONA, H. R. N. Ethical principles in the animal experimentation. *Rev. Ciênc. Farm.*, v.24, n.2, p.97-105, 2003.

MARONA, H. R. N.; LUCCHESI, M. B. B. Refining the intestinal motility test in mice to reduce animal stress. *Rev. Ciênc. Farm.*, v.24, n.1, p.79-82, 2003.

MARONA, H. R. N; LUCCHESI, M. B. B. Protocol to refine intestinal motility test in mice. *Lab. Anim*., v.38, p.257-260, 2004.

NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES (NIEHS). *Current status of in vitro test methods for identifying ocular corrosives and severe irritants*: bovine corneal opacity and permeability test method. 2006. (NIH Publication, n.06-4512). Available at: <http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm#bcop>. Accessed on: 10 jul. 2008.

NATIONAL INSTITUTES OF HEALTH, NIH. *Corrositex®*: an *in vitro* test method for assessing dermal corrosivity potential of chemicals. 1999. (NIH Publication, n.99-4495). Available at: <http://www.invitrointl.com>. Accessed on: 24 feb. 2008.

NATIONAL TOXICOLOGIC PROGRAM (NTP). Test Methods Evaluations, Dermal Corrosivity and Irritation, Corrositex®. Available at: <http://iccvam.niehs.nih.gov/methods/dermal/corrode.htm>. Accessed on: 24 feb. 2008.

NEWLY APPROVED OCULAR SAFETY METHODS REDUCE ANIMAL TESTING. National Institutes of Health. Available at: <http://www.nih.gov/news/health/jun2008/niehs-23.htm>. Accessed on: 2 aug. 2008.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT. *OECD guidelines for the testing of chemicals*: acute eye irritation/corrosion. Paris: OECD, 2002. 14 p. (OECD guidelines for testing of chemicals, 405).

POLITI, F. A. S.; SALGADO, H. R. N.; PIETRO, R. C. L. R. Caracterização de biotérios, legislação e padrões de biossegurança. *Rev. Ciênc. Farm. Básica Apl.*, v.29, n.1, p.17-28, 2008.

SAMPLE PROTOCOL CORROSITEX®. A Validated and Accepted Dermal Corrosion Test Method for Classifying Substances According to UN Packing Groups. Available at: <http://www.invitrointl.com>. Accessed on: 24 feb. 2008.

SILVA, O.; ROUGIER, A.; DOSSOU, K. G. The HET-CAM test: a study of irritation potential of chemicals and formulations (L'OREAL). *Altern. Labor. Anim.*, v.20, n.3, p.432-437, 1992.

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