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HET-CAM METHOD AS AN ALTERNATIVE MODEL TO OCULAR IRRITATION TEST FOR NATURAL PRODUCT

USO DO MÉTODO HET-CAM COMOMODELO ALTERNATIVO AO TESTE DE IRRITAÇÃO OCULAR PARA PRODUTOS NATURAIS

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Abstract

The society's pursuit of more sustainable products and natural raw materials has sparked scientific interest and prompted pharmaceutical and cosmetic industries to seek more sustainable alternatives. In 1944, Draize described the first tests for ocular irritation aimed at evaluating the degree of irritation caused by drugs, cosmetics, and other chemicals in rabbit eyes. Recently, CONCEA published the new normative resolution, No. 54, making the use of validated alternative methods with national or international regulatory acceptance mandatory. Given this context, the study aimed to evaluate the predictive potential of the alternative methodology using the chorioallantoic membrane of the chicken embryo (HET-CAM) and its modification (CAM-TBS), in order to propose alternatives for assessing the ocular toxicity of

natural products with low irritant potential. The test products used were the crude extract of sapucaia seed byproduct, a natural emulsion base, and 10% urea cream, coded as A01, A02, and A03, respectively. In the HET-CAM assay, a false-negative result was observed, indicating inferior performance compared to CAM-TBS. The same product classified as "non-irritant" in the HET-CAM assay received a "mild irritant" classification in the CAM-TBS, indicating greater sensitivity of the CAM-TBS assay compared to HET-CAM. Based on the result, CAM-TBS demonstrated better performance compared to HET-CAM. To reduce the number of false negatives in HET-CAM, it is suggested to create a low irritant potential category with a cutoff point of 0.6 to 0.9, encompassing the "non-irritant" and "mild irritant" categories, similar to the CAM-TBS category.

Keywords: Alternative Methods, Natural Products, Irritation, Animal Cruelty-free.

Resumo

A busca da sociedade por produtos mais sustentáveis e de matéria-prima natural, alavancou o interesse científico, das indústrias farmacêuticas e cosméticos a buscarem alternativas mais sustentáveis. Em 1944, Draize descreveu os primeiros testes de irritação ocular que tinham como objetivo avaliar o grau de irritação causado por medicamentos, cosméticos e outras substâncias químicas, em olhos de coelhos. Recentemente, o CONCEA publicou a nova resolução normativa, nº 54, tornando obrigatória a utilização de métodos alternativos validados e com aceitação regulatória nacional ou internacional. Diante do contexto, o estudo teve como objetivo a avaliação do potencial preditivo da metodologia alternativa da membrana cório-alantoide de ovo embrionado de galinha (HET-CAM) e de sua modificação (CAM-TBS), visando propor alternativas para avaliação da toxicidade ocular de produtos naturais com baixo potencial irritante. Os produtos utilizados para o teste foram o extrato bruto do subproduto das sementes de sapucaia, uma base natural para emulsão e o creme de ureia 10%, codificados em A01, A02 e A03, respectivamente. No ensaio HET-CAM foi observado um resultado falso-negativo, demostrando um desempenho inferior ao CAM-TBS, uma vez que o mesmo produto, classificado como "não irritante" no ensaio HET-CAM, recebeu a classificação "irritante leve" no CAM-TBS, indicando uma sensibilidade maior deste ensaio em comparação ao HET-CAM. Diante do resultado, o CAM-TBS demonstrou melhor desempenho em relação ao HET-CAM. A fim de reduzir o número de falsos-negativos no HETCAM, sugere-se criar uma categoria de baixo potencial irritante com o ponto de corte de 0,6 a 0,9 englobando a categoria “não irritantes” e “irritantes leves”, semelhante a categoria do CAM-TBS.

Palavras-chave: Métodos alternativos; HET-CAM; CAM-TBS; Produtos Naturais.

Introduction

The Draize test described in 1944 was the first method to evaluate the degree of ocular irritation that might accidentally occur to the human eyes caused by drugs, cosmetics, and other chemicals. Despite its gold-standard status, it has been substituted by alternative methods due to the creation of Law 11,794 in 2008, also known as the Arouca Law, which promotes substantial change in animal use for testing for the safety and efficacy of drugs and cosmetics. Consequently, the National Council for the Control of Animal Experimentation in Brazil (CONCEA) established a permanent chamber for alternative methods to animal use, intending to develop, validate, and certify alternative technologies and assays (Moreto et al., 2019; Anadón et al., 2014; OECD, 2012).

Alternative methods are not limited to the replacement of animals in experiments. They can be defined as procedures that aim to replace, reduce, or refine the use of animals in research to minimize animal pain and discomfort, following the principle of the 3Rs (Reduce, Reuse, and Recycle Waste) proposed by William Russel and Rex Burch (Filho, 2016). To be considered alternative methods, they must meet specific evaluation criteria, such as skin irritation, ocular irritation, acute toxicity, and cutaneous absorption (Verstraelen et al., 2013). The search for alternative methods is a global reality aimed at achieving alternatives with lower cost, greater efficacy, and greater ease of application for *in vitro* testing (Eskes et al., 2005, ICCVAM, 2006).

On January 17, 2022, the Ministry of Science, Technology, and Innovation published the new normative resolution CONCEA No. 54, using validated alternative methods with national or international regulatory acceptance mandatory. In addition, the resolution authorizes using any validated alternative method, even if not yet nationally recognized by CONCEA, boosting the use of alternative methods. This new regulation is an essential milestone for Brazil, and it encourages innovative methodologies that promote animal use reduction, replacement, or refinement, aligning the Brazilian market with global sustainability trends.

Recently, the State of Espírito Santo, through Law No. 11325 of July 12, 2021, prohibits the use of animals for the development, experiments, and testing of cosmetics, personal hygiene, perfumes, cleaning products, and their components. In addition to the ethical issues involved, *in vitro* assays aim to achieve advantages such as greater efficacy, lower cost, and greater ease of diffusion and incorporation of these methodologies by other laboratories. Therefore, it is relevant for official quality control laboratories (Eun, 2000).

Throughout the evolution of alternative methods, several methodologies have been studied to replace the Draize eye irritation test. However, only some of them

have been validated, making it necessary to study further the applicability of these *in vitro* assays (Mitjans, 2008).

The scientific literature points out some limitations related to alternative methodologies, such as the HET-CAM model, which may underestimate or overestimate *in vivo* results depending on factors such as the nature of the test substance, its irritant potential, and the presence of irreversible effects *in vivo* (Nobrega, 2012).

Although some alternative methods, such as the isolated chicken eye test (ICE) and the bovine corneal opacity and permeability test (BCOP), have been formally validated, they are not suitable for evaluating products with low irritant potential, as they have good predictive capacity only for corrosive or severely irritant products (OECD, 2012).

In this context, this study aimed to evaluate the predictive potential of the alternative methodology of the hen's egg test-chorioallantoic membrane (HET-CAM) and its modification (CAM-TBS), which assesses damage to the chorioallantoic membrane by the amount of trypan blue dye absorbed by it, aiming to propose alternatives for the evaluation of the ocular toxicity of natural products using as a test product *L. pisonis* extract.

Materials and Methods

Samples

In this study, fruits *Lecythis pisonis Cambess*., known as sapucaia, was harvested in Laranja da Terra (Latitude: 19º53’56” South, Longitude: 41º03’24 "W -00°00", West) and Viana (Latitude: 20º23'25" South, Longitude: 40º29'46" West), cities in the state of Espírito Santo in Brazil, at the experimental farm of the Capixaba Institute of Research, Technical Assistance and Rural Extension (INCAPER). A voucher specimen was deposited in the Rio de Janeiro Botanical Garden Herbarium Collection (JBRJ-Holotype) and Royal Botanic Gardens (K000600113).

Preparation of Extract

After collecting the fruits, the shells were separated from the seeds, placed in a ventilated oven at 40°C for 12 hours, and then put in a ball mill. The ethanol extract was prepared using the Soxhlet apparatus for 6 hours and then concentrated in a rotary evaporator at 40°C until the residue was. The obtained extract was kept in a vacuum desiccator for at least 48 hours to remove the solvent and then lyophilized. After that, the extract was used to prepare a natural emulsion base using 10% urea cream purchased from a store in Vila Velha - E. A01 (crude extract); A02 (natural

base for emulsion), and A03 (urea 10%), control negative (saline 0.9%), and control positive(SDS 1% for moderate irritation), (NaOH 0,1M for severe irritation), and tested without dilution.

HET-CAM Assay

For the experiment, 3 eggs and control groups were used per tested product. On the tenth day of incubation, the eggshell was removed around the air chamber, exposing its membrane. The membrane was gently extracted, displaying the chorion-allantoic membrane, on which 300 µL of the product was applied. After 20 seconds of contact, the product was removed, and the chorion-allantoic membrane was washed with isotonic saline solution at 37.0°C. For 5 minutes, the chorion-allantoic membrane was examined, and the observed physiological reactions were graded according to their appearance time, as indicated in Table 2. This assay was performed according to the methodology of The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) No. 07-4517. The final classification of products according to their potential for irritability in the HET-CAM assay is described in Table 3.

CAM-TBS Assay

Initially, this methodology is similar to the HET-CAM assay, where 3 eggs were also used, which on the tenth day of incubation, were exposed to the chorion-allantoic membrane. A silicone ring was placed on this membrane to delimit the treated area, and 300 µL of the product was added to the chorion-allantoic membrane. After 20 seconds, the area was washed with distilled water at room temperature. After removing the product, 500 µL of 0.1% (w/v) trypan blue dye solution was applied and left to act for 1 minute. Then, the dye was removed by washing the treated area with a 3 mL syringe containing distilled water for 20 seconds. With scissors and tweezers, the treated membrane area was extracted and placed in a falcon tube containing 5 mL of formamide, which was centrifuged at a speed of 3,200 rpm for approximately 10 minutes. The supernatant was transferred to a new tube, and the optical density of the resulting solution was measured at 595 nm. The results were analyzed according to the methodology described in the CAM-TBS assay. A plate reader performed the 96-well plate and the reading at a wavelength of 595 nm. The amount of trypan blue absorbed by the chorioallantoic membrane was calculated using the following equation 1. The dye calibration curve was made using solutions of trypan blue in formamide at concentrations of 10^-6, 10^-5, and 5 × 10^-5 mol/L, read on a spectrophotometer at a wavelength of 595 nm. This assay was performed according to the methodology described in INVITTOX

protocol no. 10818, and the products were classified according to Lagarto's proposal (2006), as shown in Table 4.

$$\text{absorbed dye} = \frac{(\text{absorbance x 5 nmol})}{1{,}000}\ x\ 10\text{^}9 \quad (1).$$

Statistical analysis

They were compared using contingency tables to determine the sensitivity, specificity, and accuracy of the HET-CAM and CAM-TBS models.

Results

Based on the means obtained from triplicates in the HET-CAM and CAM-TBS assays, each sample could be classified according to its specific methodology. Table 5 shows the results of this classification, as well as the average and classification values obtained, which ranged from 0 to 19 for the HET-CAM mean values and < 7nm for the dye absorption range in the CAM-TBS.

However, results indicated that this test still has a specificity and accuracy of 100% compared to CAM-TBS.

CAM-TBS presented a higher sensibility classifying the product A03 as "mild irritant". The same product received in the HET-CAM assay had a classification of "not irritant." The CAM-TBS demonstrated better performance (100% sensitivity) compared to HET-CAM (66.67%). Sample A03 (urea 10%) observed a false-negative result for the HET-CAM assay. However, results demonstrated in Table 5 indicated that this test still has a specificity and accuracy of 100% compared to CAM-TBS, Table 5.

Discussion

The term "alternative method" is associated with the principles of the 3Rs described by Russell and Burch in 1959 (Filho, 2016) and suggested due to concerns for animal welfare (Anadón et al., 2014). Thus, ethical reasons combined with scientific needs have stimulated the development and validation of several alternative methods aimed at replacing the Draize test and reducing animal suffering for research purposes (Cazarin; Correa; Zambrone, 2004; Scheel et al., 2011; Verstraelen et al., 2013; Parascandola, 1991).

When *in vivo* assays cannot be replaced by a single alternative method, such as in the case of the Draize eye irritation test, it becomes significant to consider the development of analyses that include a variety of assays. Considering different aspects and biological models should be taken into consideration for the evaluation of the effects of the tested product. Additionally, the combination of different assays can help improve the accuracy and reliability of the obtained results (Costa, 2011; Donahue, 2011).

For ingredients with strong irritant potential, models such as the BCOP and ICE are available and have good regulatory acceptance (Esac, 2009). However, evaluating finished products with low irritant potential remains challenging, as it is sometimes easy to identify which assays are most appropriate for each product. In these cases, a careful and thoughtful approach is fundamental to ensuring the safety and efficacy of the products.

The HET-CAM assay determines macroscopic changes in the chorion-allantoic membrane resulting from applying ingredients with irritant potential. However, this method has been criticized for its qualitative outcome and subjectivity in the readings obtained for hyperemia, hemorrhage, and coagulation/opacification over 5 minutes. In addition, the subjectivity of the HET-CAM outcome makes interlaboratory reproducibility difficult, which hurts its potential for diffusion and transfer to many laboratories (Garcia, 2004; Lagarto, 2006).

Given the negative impact on the HET-CAM assay, the CAM-TBS assay was developed to overcome its limitations by offering a quantitative readout by measuring trypan blue dye absorbed by the exposed chorioallantoic membrane. This dye is widely used in laboratories, and its determination is simple and reproducible (OECD, 2012).

A study conducted by Lagarto and colleagues evaluated 21 samples in the CAM-TBS assay, including chemicals and cosmetic products, finding a more robust correlation coefficient for isolated chemicals than for finished products. The authors proposed a cut-off point for the CAM-TBS model classification (cut-off = 7.0), aggregating "non-irritant" and "mild irritant" samples into a single class. This classification is the most appropriate for comparing assays, leading to a sensitivity of 100% under the proposed experimental conditions. In contrast, the HET-CAM led to false-negative results and a sensitivity of only 66.67%. It is important to note that even with the difference found in terms of sensitivity between HET-CAM and CAM-TBS, the precision and specificity of both assays were 100%, indicating that the CAM-TBS assay significantly supports the efficacy of HET-CAM, indicating that the classification criteria adopted in this study appear to be the most appropriate for products with low irritant potential.

Conclusion

The CAM-TBS demonstrated better performance (sensitivity of 100%) compared to the HET-CAM (66.67%). In order to reduce the number of false negatives in the HET-CAM, it is suggested to create a category of low irritant potential with a cutoff point of 0.6 to 0.9, encompassing the categories of "non-irritants" and "mild irritants," similar to the CAM-TBS category. The CAM-TBS, in turn, presents advantages over the assays, due to its quantitative outcome and the classification criteria adopted by Lagarto et al. (2006), appearing to be the most suitable method for products with low irritant potential. It can be considered as an alternative method of choice for the classification of irritation in natural products.

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