

A proposal of an alternative *in vitro* method for the evaluation of the safety of shampoos

Proposta de método alternativo *in vitro* para avaliação da segurança de shampoos

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RIALA6/957

Azevedo, J. C. de; Cruz, A. S. e Pinto, T. de J. A - A proposal of an alternative *in vitro* method for the evaluation of the safety of shampoos. **Rev. Inst. Adolfo Lutz**, 62(3): 189 - 193, 2003.

ABSTRACT. Although tests using animals are still demanded in the evaluation of shampoos, there is a strong evidence of a tendency for replacing them by *in vitro* systems. This study aims at defining conditions for the application of a method using NCTC clone 929 cells, as an alternative. Procedures employed to test non diluted samples of shampoos for adults resulted in irritation (in the *in vivo* method); the same result was not observed when samples diluted to a 25% concentration were tested. In the *in vitro* tests, the same shampoos presented a perfect correlation of results when not diluted (cytotoxic) and when diluted to a 0.1% concentration (non cytotoxic). When the shampoos for children use were tested, the only case of irritation happened with one of the 4 non-diluted shampoos. The absence of cytotoxicity was observed in the *in vitro* test, after diluting all the samples to 1% and 0.1%. It is possible thus to use the *in vitro* test instead of the *vivo* one, if preceded by a careful validation with emphasis on the concentration of the product.

KEY WORDS. eye irritation, cell culture, cytotoxicity, Draize

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INTRODUCTION

The eye irritation test in rabbits is the method currently used to evaluate the hazard or safety of substances introduced into the eyes or used in their close proximity⁴. To reduce the need for animal testing, as well as to establish a more rapid and practical method, "in vitro" assays are being developed as an alternative to the rabbit test^{1,8}.

The development of an *in vitro* assay is a great challenge. The tendency is to use a single selected cell type instead of the eye, which is a complex biological system.

Several points have been related to the biological test performed in an animal organ, such as a rabbit eye. For instance, the rabbit eye has a nictitating membrane, non existing in the human eye; a rabbit eye produces, in an aggressive situation less tears than the human one does. When an irritating material is placed in the rabbit eye the tears, at first, are usually clear. Within 24 hours, however, a viscous milky secretion is seen which appears to come from under the nictitating membrane². On the other hand, in the Draize test the product under evaluation is applied without previous dilution. If we consider the practical use of a shampoo, we have to take into account that, except for an accident, only residual quantities of the product will remain on the eye surface. Considering the above facts, this study aims to define details of experimental conditions for a cell culture system which could be used instead of the current Draize score.

MATERIAL AND METHODS

The test material used in this study consisted of 8 different shampoos, 4 for adults (A, B, C and D) and 4 for children (E, F, G and H), each one of them represented by three batches.

They were assayed by the conventional Draize method, according to the Food and Drug Administration (FDA) protocol³, using six rabbits. The samples were tested directly and after water dilution (25%, 5%, 1% and 0.1%). The instillation of samples was in a 0.1 mL volume, in one of the animal eyes, being the other one the control. The observation of the reactions involving cornea, iris and conjunctiva (chemosis, redness) happened after one, two, three and seven days.

The same shampoos, with equal concentrations, were also tested by an *in vitro* using the selected NCTC clone 929 cell line grown in Petri dishes in a concentration of 3×10^5 cells per mL in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (GibcoTM, USA). The Petri dishes were put in an atmosphere of 5% CO₂ in air for 48 hours at 37°C with the purpose of getting a cell monolayer. This culture medium was replaced by a 5 ml volume of a solution of 1.8% agarose, containing 0.01% of neutral red mixed in equal parts with Eagle's minimum essential medium without serum. After wetting filter-paper discs (FramexTM) in the samples, each one of them was deposited on the agar surface of six dishes and then put again in a atmosphere of 5% CO₂ in air for 24 hours at 37°C. As positive

Table 1. Draize test and tytototoxicity assay results applied to shampoos for adult use performed in several dilutions

Sample	Batch	100%		25%		5%		1%		0.1%	
		in vivo	in vitro								
A	1	I	C	NI	C	NI	C	NI	NC	NI	NC
	2	I	C	NI	C	NI	C	NI	NC	NI	NC
	3	I	C	NI	C	NI	C	NI	NC	NI	NC
B	1	I	C	NI	C	NI	C	NI	C	NI	NC
	2	I	C	I	C	NI	C	NI	C	NI	NC
	3	I	C	NI	C	NI	C	NI	C	NI	NC
C	1	I	C	NI	C	NI	C	NI	C	NI	NC
	2	I	C	NI	C	NI	C	NI	C	NI	NC
	3	I	C	NI	C	NI	C	NI	C	NI	NC
D	1	I	C	NI	C	NI	C	NI	C	NI	NC
	2	I	C	NI	C	NI	C	NI	C	NI	NC
	3	I	C	NI	C	NI	C	NI	C	NI	NC

I - Irritant / C - Cytotoxic / NI - Non Irritant / NC - Non Cytotoxic

Table 2. Draize test and cytotoxicity assay results applied to shampoos for children use performed in several dilutions

Sample	Batch	100%		25%		5%		1%		0.1%	
		in vivo	in vitro								
E	1	NI	C	NI	C	NI	NC	NI	NC	NI	NC
	2	NI	C	NI	C	NI	NC	NI	NC	NI	NC
	3	NI	C	NI	C	NI	NC	NI	NC	NI	NC
F	1	NI	C	NI	C	NI	C	NI	NC	NI	NC
	2	NI	C	NI	C	NI	C	NI	NC	NI	NC
	3	NI	C	NI	C	NI	C	NI	NC	NI	NC
G	1	I	C	NI	C	NI	C	NI	NC	NI	NC
	2	I	C	NI	C	NI	C	NI	NC	NI	NC
	3	I	C	NI	C	NI	C	NI	NC	NI	NC
H	1	NI	C	NI	C	NI	C	NI	NC	NI	NC
	2	NI	C	NI	C	NI	C	NI	NC	NI	NC
	3	NI	C	NI	C	NI	C	NI	NC	NI	NC

I - Irritant / C - Cytotoxic / NI - Non Irritant / NC - Non Cytotoxic

and negative controls, 1N sodium hydroxide and 0.9% NaCl solutions were respectively used. The presence of leachable toxic substances was manifested by the loss of dye from the cells as well as by the lysis of the cells within the zone, near the disc. The toxic response is given as negative or positive, taking into account a response index based on the size of the diffusion (decolorized) zone¹⁰.

RESULTS

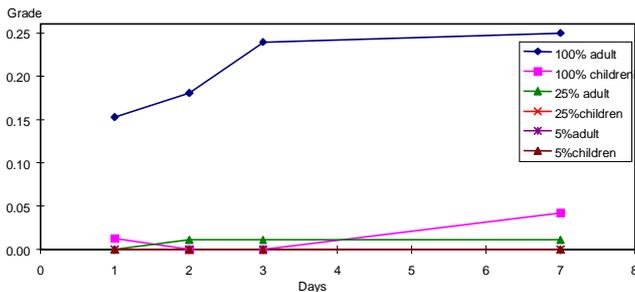


Figure 1. Cornea grades averages (shampoos for adult and children use), each day

It is possible to observe in Table 1 that all the shampoos for adults are, according to the Draize score, considered as irritant when tested without dilution. After dilutions to 25% only a batch has persisted irritant. However, below this concentration all of them changed to a non irritant score.

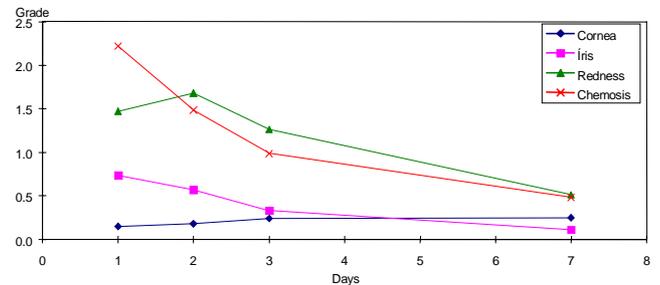


Figure 2. Reactions grade of each eye structure, referring to shampoos for adult use, without dilution

In vitro tests only diluted until 1% (Table 1) are considered, it is possible to observe a partial correlation with the rabbit tests. Nevertheless, in the case of dilution absence or a 0.1% concentration a total correlation of results was observed.

In the case of shampoos for children (Table 2) the absence of ocular irritation was detected in three of four products, after being tested without dilution. When samples were diluted to 25%, all of them were considered non irritant. As for the *in vitro* tests from a 5% concentration on, one product presented non cytotoxic effect, as verified in all samples tested with a concentration of 1% and 0.1%. Figure 1 shows the evolution of cornea reactions, according to increasing concentrations. A higher level of cornea injuries can also be observed. Figure 2 shows different levels of reactions in the eye structures produced by shampoos for adult use. The values presented in the Figure 1 and Figure 2 were calculated employing the average of grades for ocular lesions described in Code of Federal Regulation³.

DISCUSSION

The development of *in vitro* assays falls into 2 major categories: screens and replacements, whose demand of information is usually considerably more relevant. The informational demands are usually less for an assay used as a screen than for a replacement⁶.

This work defends both of them according to different steps of its development. After the introduction of a screening step, a validation program, demanding even a higher number of animals, must be started, in case the replacement of methods is desirable. We can also consider that even if some researches have compared the result of the average Draize score, it is very interesting to evaluate score irritation responses for each area of the eye separately, as an independent observation^{7,9}. Figure 2 demonstrates that it is really interesting to isolate each reaction and Figure 1 also helps to confirm the importance of testing the

non diluted shampoos, when in animal assay⁵. The cornea of the rabbits, which constitutes a protective barrier for the internal structures, suffered the most serious damage, mainly in situations of “pannus” and opacity. Comparing this reaction with the lysis of NCTC clone 929 lineage, one of the advantages of working with a *in vitro* system becomes clear.

The evaluation of the results obtained (Tables 1 and 2) enables us to verify the higher sensibility of the *in vitro* test, which induces us to valorize the parallel between the test of the non-diluted samples using animals and the diluted ones, using cells. Thus we may consider a correlation, represented by a dilution index of 1% or 0.1%. We can conclude that it is possible to apply the *in vitro* test instead of the *in vivo* one, after a careful validation, facing the different characteristics of the systems.

RIALA6/957

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RESUMO. Embora os testes empregando animais sejam os preconizados para a avaliação da segurança de xampus, tem-se buscado sua substituição por métodos *in vitro*. Este trabalho descreve a avaliação da segurança de amostras de xampus de uso infantil e adulto, diluídos e não diluídos, por meio de método alternativo empregando a linhagem celular NCTC clone 929. Os xampus de uso adulto promoveram irritação quando testados sem diluição, sendo que somente um lote ainda se mostrou irritante quando testado diluído a 25%. Verificou-se perfeita correlação entre os resultados obtidos pelos métodos *in vivo* e *in vitro* para amostras não diluídas e quando diluídas a 0,1%. Dos diferentes tipos de xampus de uso infantil, apenas uma amostra apresentou irritação quando empregada metodologia *in vivo*. Ao avaliar estas mesmas amostras pelo método *in vitro*, observou-se ausência de citotoxicidade, para todas as amostras, a partir das diluições a 1% do produto. Portanto, para utilização da metodologia *in vitro* como alternativa à metodologia *in vivo*, faz-se necessário criteriosa validação considerando a necessidade de diluição do produto.

PALAVRAS –CHAVE. irritação ocular, cultura celular, citotoxicidade, Draize

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Recebido em 20/03/2003; Aprovado em 08/10/2003