Acta Derm Venereol (Stockh) 1997; 77: 26–28

Use of XTT-assay to Assess the Cytotoxicity of Different Surfactants and Metal Salts in Human Keratinocytes (HaCaT)

A Feasible Method for In vitro Testing of Skin Irritants

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Because of the increasing need of reliable skin irritation tests and in order to reduce the number of animal experiments, in vitro alternatives have to be developed. We studied four surfactants and five metal salts for their cytotoxic potency in HaCaT cells, a spontaneously immortalized human kertinocyte line. The endpoint used to assess cellular viability was metabolization of the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide).

The tested substances revealed a significant rank order of their cytotoxicity at an exposure time of 24 h. It was 1) benzalkonium chloride, 2) sodium lauryl sulphate, and 3) Tween 20 (polyoxyethylene sorbitanmonolaurate) and Tween 80 (polyoxyethylene sorbitanmonooleate), for the surfactants; and 1) potassium bichromate, 2) copper sulphate, 3) cobalt chloride and palladium chloride, and 4) nickel sulphate, for the metal salts. There is an excellent correlation to the rank order of their known irritative potency in vivo.

Being practicable and effective, the presented XTT-assay on HaCaT cells would be well suitable for an initial orientating screening of substances, subsequently followed by irritation tests directly in humans. ***Key words: skin irritation; keratinocyte culture.***

(Accepted June 14, 1996.)

Acta Derm Venereol (Stockh) 1997; 77: 26–28.

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"Can this substance possibly damage the skin?" is an ever recurring question when new chemical products are being developed. Up to this day animals have been used to assess cutaneous irritation (1). But the accuracy and reliability of these methods in relation to human skin have been called into question (2, 3), and there must also be objections for ethical reasons. For many years efforts have been made to develop alternative testing methods (4–6). Viability tests in cultured human keratinocytes have proved to be a promising in vitro model for predicting skin irritation.

In this study we employed the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide)-assay (7), a tetrazolium/formazan assay, to measure cytotoxicity in HaCaT cells, a spontaneously transformed human epithelial cell line from adult skin, which is immortal, nontumorigenic and maintains full epidermal differentiation capacity, established by Boukamp et al. in 1988 (8).

We chose four surfactants, benzalkonium chloride, sodium lauryl sulphate, Tween 20 (polyoxyethylene sorbitanmonolaurate) and Tween 80 (polyoxyethylene sorbitanmonooleate), and five metal salts, nickel sulphate, potassium bichromate, cobalt chloride, copper sulphate and palladium chloride, for cytotoxicity testing. Surfactants frequently cause irritant contact dermatitis. Metal salts are potent allergens, but they are also known to have a toxic effect on the skin, which, however, has rarely been investigated by in vitro or in vivo models.

MATERIAL AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin (100 U/ml and 100 µg/ml), serum-free keratinocyte medium (K-SFM), Dulbecco's phosphate buffered saline (PBS), trypsin-EDTA and trypan blue 0.4% were obtained from Gibco and EDTA from Boeringer Mannheim. The XTT-assay kit "EZ4U–EASY FOR YOU–non-radioactive cell proliferation and cytotoxicity assay" was purchased from Biozol. The test substances benzalkonium chloride, sodium lauryl sulphate, potassium bichromate, nickel sulphate and copper sulphate came from Laborchemie Apolda, Tween 20 from Sigma, Tween 80 from Serva and cobalt chloride and palladium chloride from Merck-Schuchardt. The surfactants and metal salts were solubilized in distilled water and serial dilutions were made using the unit "mol/l". HaCaT cells were kindly provided by Professor Fusenig (German Cancer Research Center, Heidelberg).

Keratinocyte culture

HaCaT cells were cultured in DMEM supplemented with 10% FCS and 2% penicillin/streptomycin as a monolayer in tissue culture flasks at 37°C and 5% CO_2. Following trypsinisation and cell counting, cells were seeded into 96-well microtiter plates, each well containing 180 µl of cell suspension in K-SFM.

XTT-assay

First of all we determined the relationship between cell number and formazan production. For this purpose cell suspensions with increasing cell density were seeded into plates and cultivated for 3, 6, 24, 69 or 120 h, respectively. Twenty-five microlitres of the XTT-solution were added to each well. Following 3 h incubation, absorbance was measured at 450 nm in an ELISA-reader (Photometer Reader 400 SF, Medgenix, SLT Labinstruments). The mean absorbance of two blanks (no cells in the well) was automatically subtracted from all values.

Cytotoxicity

After being seeded and becoming adherent, cells in each well were exposed to 20 µl of the solubilized noxae in different concentrations for 3, 6 or 24 h, respectively, using separate plates for each exposure time, followed by performing the XTT-assay as described above. Three wells on each plate were supplied with the same concentration of the noxa, and every experiment was repeated three times independently. All absorbance values were expressed as per cent of controls obtained from wells which were exposed to distilled water instead of noxa. For all tested substances dose-response curves were constructed for the three different exposure times, from which the IC_{50} (IC = inhibitory concentration) can be determined, the concentration resulting in 50% XTT reduction. We used the *t*-test for statistical evaluation of the results.

 ISSN 0001-5555

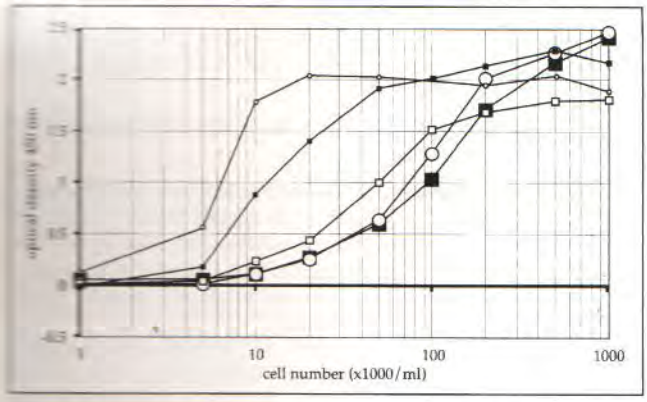

Fig. 1. XTT-assay: dehydrogenase activity of HaCaT cells depending on seeded cell number and duration of cultivation. ■ 3h ○ 6h □ 24h ● 69h ◦ 120h

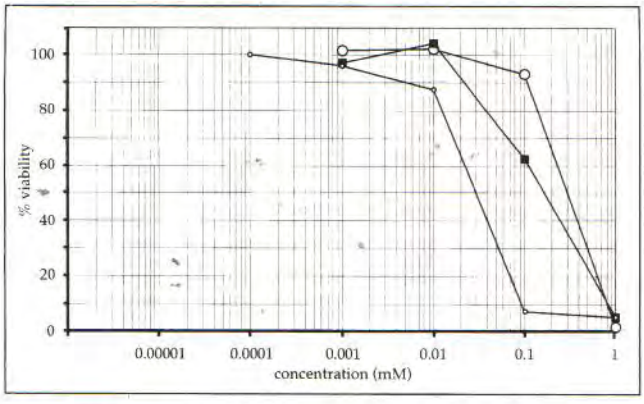

Fig. 2. XTT-assay: dehydrogenase activity of HaCaT cells treated with sodium lauryl sulphate. ○ 3h ■ 6h ◦ 24h exposure time

Skin irritation tests in vivo

Solutions of the four surfactants were applied to the volar forearms of 4 healthy adult human volunteers by moistening the test areas with a cotton bud every 30 s for 30 min daily. Responses were read immediately as well as 2 h and 24 h after application. Erythema of the test area was regarded as a positive reaction. If there was no positive result after 24 h, the test was repeated at the same area. This open repetitive patch test according to Burckhardt-Schmid (9) closely imitates what actually happens to the skin when exposed to a substance and takes into account the cumulative effect of irritation and the repair mechanisms of the skin. In the first series of experiments at the left forearm the surfactants were tested in 1,000 fold $IC_{50/6\,h}$, in the second series at the right forearm in 5,000 fold $IC_{50/24\,h}$, for 7 days.

RESULTS

Establishing the assay and choice of cell number

The relationship between absorbance and seeded cell number as well as cultivation time is shown in Fig. 1. The curves flatten with rising cell concentration in the well. However, there is proportionality at low cell numbers, up to 10^5/ml, and short cultivation times, up to 24 h. We chose cell numbers of 4×10^4/ml for 6-h and 24-h exposure time and 10^5/ml for 3-h exposure time to be within the proportional range and obtained control values of absorbance between 0.5 and 1.0.

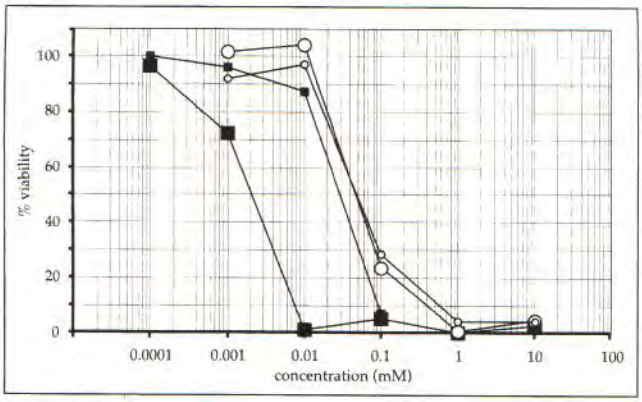

Fig. 3. XTT-assay: dehydrogenase activity of HaCaT cells treated with different surfactants (exposure time 24h) ■ benzalkonium chloride ○ Tween 80 ● sodium lauryl sulphate ◦ Tween 20

Cytotoxic effect in vitro

All tested surfactants and metal salts showed a cytotoxic effect depending on their concentration and exposure time, as expressed by decreased absorbance values. Fig. 2 shows the dose-response curves for sodium lauryl sulphate, as an example. Comparing the cytotoxic potency of the tested substances at equal exposure times, we found a significant rank order at 24 h. It is 1) benzalkonium chloride 2) sodium lauryl sulphate, and 3) Tween 20 and Tween 80 (no significance), for the surfactants (Fig. 3); and 1) potassium bichromate, 2) copper sulphate, 3) cobalt chloride and palladium chloride (no significance), and 4) nickel sulphate, for the metal salts (Fig. 4).

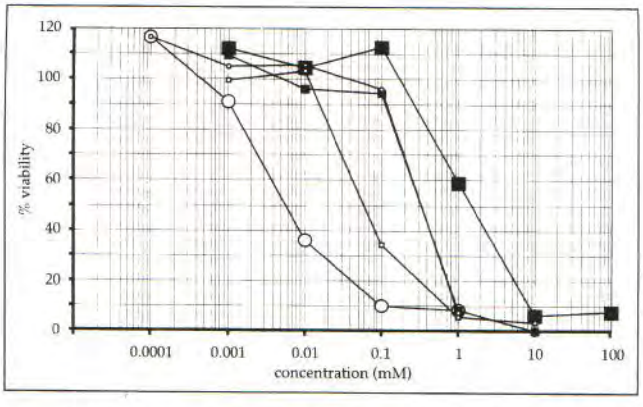

Fig. 4. XTT-assay: dehydrogenase activity of HaCaT cells treated with different metal salts (exposure time 24h) ■ $NiSO_4$ ○ $K_2Cr_2O_7$ ● $CoCl_2$ ◦ $PdCl_2$

Irritative effects of surfactants in vivo

The Tweens did not produce any irritation within 7 days of testing. However, all volunteers responded not later than at the third day to sodium lauryl sulphate and at the same day or two days later to benzalkonium chloride.

DISCUSSION

We presented an assay system as a microculture method to evaluate the cytotoxic potency of surfactants and metal salts.

Using the permanent HaCaT cell line, which in its characteristic features still largely resembles normal human keratinocytes (8), gives a high standardization of experimental conditions as well as a good prediction referring to human skin. The XTT-assay is simple and rapid to perform, comparatively inexpensive and not dangerous for man or environment.

However, there are some shortcomings concerning its stability and reproducibility. Tetrazolium reduction is influenced by a number of metabolic and other factors, which accumulate during the experiment and may evoke errors (10–12).

The rank order of the four surfactants established by the XTT-assay in HaCaT cells corresponds not only with the in vitro results of many investigators (13–15), but also exactly with the rank order of their irritative potency in vivo (16, 17). There are only a few such investigations on the irritative effect of metal compounds. Their in vivo rank order is deducible from the patch test concentrations currently recommended by the "International Contact Dermatitis Research Group (ICDRG)": 1) potassium bichromate (0.5%), 2) copper sulphate, cobalt chloride and palladium chloride (all 1%), and 3) nickel sulphate (5%) (18). The same rank order was established by the presented in vitro method. Thus, concerning the rank order of the nine tested noxae, there is an excellent conformity between our in vitro results and the known in vivo characteristics.

The parameter "decreasing dehydrogenase activity" served as a dimension of cell damage, and cell damaging again was employed to predict the irritative potency of a substance. However, such a simplification hardly applies to the complex pathomechanisms of skin irritation. Our volunteer studies confirm that fact: the surfactants were tested in a fixed multiple of their in vitro IC_{50}, i.e. the same concentration rate at which they had caused the identical in vitro effect of diminishing cellular metabolism by 50%. Despite so to speak "isotoxic" concentrations the in vivo effect is not identical: sodium lauryl sulphate gives the highest, benzalkonium chloride a comparable to some extent lower irritative effect, and the Tweens do not cause irritation at all. These differences might be ascribed to the poor penetration ability of the Tweens and the very good one of sodium lauryl sulphate.

Thus, a quantitative comparison between in vitro and in vivo results, i.e. concluding from quantitative in vitro results the degree of difference in the irritative potency of unknown substances, seems to be impossible. A substance 1 being more cytotoxic than substance 2 in vitro can solely be predicted to be also more irritating than substance 2 in vivo. Availing oneself of reference substances of the same class as a standard of comparison would be necessary for testing of unknown compounds, e.g. new surfactants.

Our experimental results indicate that the XTT-assay in HaCaT cells may be used as a screening method, possibly as a component of a battery of different methods comprising different aspects of skin irritation like penetration and cytotoxicity. If expected to be well tolerated, the substance could be subsequently tested for skin irritation directly in humans.

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