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Comparison of various methods to analyse toxic effects in human skin explants: Rediscovery of TTC assay

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ABSTRACT

Skin explants are a suitable model which can replace dermatological experiments on animals or human volunteers. In this study, we searched for a fast, cheap and reproducible method for screening skin explant viability after treatment with UVA radiation or/and chemical agents. We compared frequently used methods: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) and lactate dehydrogenase (LDH) activity assay with a rarely used 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) assay for the evaluation of UVA radiation and/or chlorpromazine and 8-methoxypsoralen effect as model agents. Histological analysis of skin explants was also performed by a simple haematoxylin-eosin method. Only the TTC assay was able to show the toxicity of model agents in a dose- and concentration-dependent manner. LDH assay was partially able to demonstrate results comparable to the TTC method, however, the agents' effect was less pronounced. The MTT and NR assays completely failed in the evaluation. Haematoxylin-eosin staining showed discrete structural changes in samples treated with UVA alone and CPZ + UVA, but only after 48 h. Therefore, the method is not useful for screening of toxic or phototoxic effects either. In conclusion, the TTC assay was the most suitable for the evaluation of toxicity or phototoxicity in ex vivo skin.

1. Introduction

The skin protects our body against physical, chemical and biological damage. Therefore, the study of physiological and pathological processes that occur in skin tissue is important for the prevention and treatment of skin damage. Likewise, evaluation of the side effects of compounds used in the chemical, pharmacological, dermatological and cosmetic industries is important knowledge for both manufacturers and users. The skin of human volunteers and mammals are ideal models for dermatological research, as they allow investigations that are close to reality. However, their use is restricted for ethical reasons. Therefore, 3D models like reconstructed epidermis or reconstructed skin and skin explants have been established to better simulate the processes in skin tissue compared to 2D models (single cell cultures, co-cultures) [1]. Reconstructed models (organotypic skin cultures) represent a powerful preclinical tool for dermatological research but they have various limitations. Compared to the native skin the cultures have a simpler microstructure such as thinner stratum corneum (reduced barrier function) and no dermal papillae, they do not contain all types of skin cells and their preparation is demanding and time-consuming [1,2]. However, intensive research in this area strives for improvement of qualities of reconstructed skin models. Currently skin explants seem to be the most useful system as the tissues contain most types of skin cells such as keratinocytes, melanocytes, fibroblasts and Langerhans cells, and also all components of the extracellular matrix such as the dermal protein fibres elastin and collagens. Skin explants can be easily prepared from donor tissues and fragments are stable for at least 10 days in culture. Some studies suggest even longer usability [3,4]. Skin explants can be used in various areas, such as for evaluating radiation-induced damage and protection against it [5,6], skin wound healing [7], sensitization [8], contact dermatitis [9] or skin irritants [10].

A number of organic compounds containing benzene or heterocyclic ring(s) have been found to be activated by sunlight and provoke a phototoxic response in the skin [11]. Photo-activated molecules can

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride; TPF, 1,3,5-triphenylformazan; CPZ, chlorpromazine; 8-MOP, 8-methoxypsoralen; NR, neutral red; LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; Ham-F12, Ham-F12 Nutrient Mixture; FCS, heat-inactivated foetal calf serum; PBS, phosphate buffered saline; PBS-G, PBS supplemented with glucose

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directly or indirectly react with endogenous molecules and on the basis of the photobiochemical interactions and clinical signs, they can elicit harmful effects including phototoxicity (photoirritation) via oxidative damage to cellular lipids and proteins, photogenotoxicity via DNA damage, and photoallergy via the formation of photoantigens [12]. The primary assay recommended for identifying phototoxicity/photoirritancy is an in vitro method, the 3T3 Neutral Red Uptake Phototoxicity Test (3T3NRUPT) [13]. This method uses a Balb/c 3T3 cell line (Balb/c), clone A31, derived from mouse embryos by Aaronson & Todaro [14]. The test principle is a comparison of compound cytotoxicity tested in the presence and absence of exposure to a non-cytotoxic dose of UVA light. Cytotoxicity is evaluated as the uptake of the weak cationic dye neutral red accumulating in the lysosomes of viable cells, measured 24 h after treatment with the test chemical and irradiation [13]. Due to its sensitivity, specificity and robustness, the 3T3 NRU is considered the core test besides additional tools such as reconstructed human skin models that uses 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, interleukin-1 assay or possibly lactate dehydrogenase (LDH) assay as the endpoint, or the human in vivo photopatch test [15].

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC), a cellular redox indicator, is one of the most frequently used compounds in histochemical staining methods. It indicates the state of cellular metabolism, therefore it is used to differentiate between metabolically active and inactive part (s) of tissues. TTC is predominantly reduced by Complex I of the respiratory chain and more slowly by other dehydrogenases [16]. Colourless TTC is reduced by mitochondrial dehydrogenases to red 1,3,5-triphenylformazan (TPF) in living cells. At the same time, it stays in the form of white TTC in areas of necrosis, since these enzymes have been either denatured or degraded. TTC reduction is commonly used as a quantitative method in the evaluating the viability of tissues or bacterial colonies. In humans and mammals, TTC is mostly used in the identification of myocardial and brain infarctions or skeletal muscle ischemia [17–19]. The use of TTC for assessing skin tissue viability was once described sixty years ago [20]. Now it is not practically used for this purpose except for Shyu et al., who recently used TTC for evaluating the survival of the abdominal skin flap in microsurgery [21]. In this study, we employed TTC for evaluating the viability of skin explants that were physically (UVA radiation) or photo-chemically (chlorpromazine (CPZ) or 8-methoxypsoralen (8-MOP) combined with a non-toxic dose of UVA radiation) damaged. The TTC assay was compared with common viability tests, including MTT assay, neutral red (NR) assay and LDH activity.

2. Material and Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), Ham-F12 Nutrient Mixture (Ham-F12), heat-inactivated foetal calf serum (FCS), stabilised penicillin-streptomycin solution, amphotericin B, hydrocortisone, adenine, insulin, epidermal growth factor, 3,3′,5-triiod-L-thyronin, trypsin, ampicillin, trypsin-EDTA (0.25%), MTT, NR, TTC, NADH, pyruvate, CPZ, 8-MOP, haematoxylin, eosin and all other chemicals were purchased from Sigma-Aldrich (USA).

2.2. Skin Explants Preparation and Cultivation

Skin explants were prepared from the skin of healthy adult donors. Breast tissue specimens were obtained from women undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital in Olomouc). The use of skin tissue complied with the Ethics Committee of the University Hospital in Olomouc and Faculty of Medicine and Dentistry, Palacký University, Olomouc (date: 6.4.2009, ref. number: 41/09). All patients had given their written informed consent.

Before use, the skin fragments were washed three times in phosphate buffered saline (PBS) containing antibiotics (penicillin (500 mg/ml), streptomycin (500 U/ml) and amphotericin B (1.25 mg/ml)). The skin was then washed with PBS and cut into pieces of approximately 0.5 × 0.5 cm. Skin explants were put into Petri dishes. After a few minutes stabilization medium was applied so that their surface stayed in contact with the air. The culture medium consisted of DMEM and Ham-F12 (1:3) supplemented with FCS (10%; v/v), penicillin (100 mg/ml), streptomycin (100 U/ml), amphotericin B (0.125 mg/ml), hydrocortisone (0.8 μg/ml), adenine (24 μg/ml), insulin (0.12 U/ml), epidermal growth factor (1 ng/ml) and 3,3′,5-triiod-L-thyronin (0.136 μg/ml). Explants were cultured in a humidified atmosphere with CO_2 (5%; v/v) at 37 °C. The medium was changed as required.

2.3. Evaluation of Viability of UVA-irradiated Explants

Skin explants were cultured on 10-cm Petri dishes before their use in an experiment. The medium was removed, the tissues were washed twice with PBS and then PBS supplemented with glucose (PBS-G; 1 mg/ml) was applied. The explants were exposed to UVA radiation (20, 40 or 60 J/cm^2) using a solar simulator SOL 500 equipped with an H1 filter transmitting wavelengths of 315–3000 nm. Spectral distribution of the SOL unit in comparison with natural sunlight is shown on Fig. 1. The UVA output was measured before each experiment with a UVA meter (Dr. Hönle UV Technology, Germany). Non-irradiated tissues were kept in the incubator. Skin specimens were then transferred onto 24-well plates and culture medium was applied. Viability was evaluated immediately, 4, 24 or 48 h after UVA exposure. One skin sample per each UVA dose was taken for histopathological examination. Culture medium was collected for the LDH assay and skin explants were then used for NR, MTT and TTC assays. All dyes (NR, MTT and TTC) were diluted in PBS-G. The final concentration of NR was 0.01%, of MTT 0.05% and of TTC 0.5%. Skin explants on 24-well plates were treated with individual solutions (0.5 ml/well) for 2 h. After incubation, the tissues were washed with PBS, dried using gauze and weighed. Retained NR was extracted with 0.5 ml of acetic acid (1%, v/v) in methanol (50%, v/v) overnight at 4 °C. The purple (from MTT) or red (from TTC) formazan produced was extracted with 0.5 ml of dimethyl sulfoxide with NH_3 (1%, v/v) overnight at 4 °C. The absorbance of extracted dyes was measured on a microplate reader (Sunrise Remote; Tecan, Austria) at 540 nm (NR and MTT) or at 485 nm (TTC). The activity of LDH released into the medium was measured spectrophotometrically by the disappearance of NADH during the LDH-catalysed conversion of pyruvate to lactate as a decrease in absorbance at 340 nm [22]. The

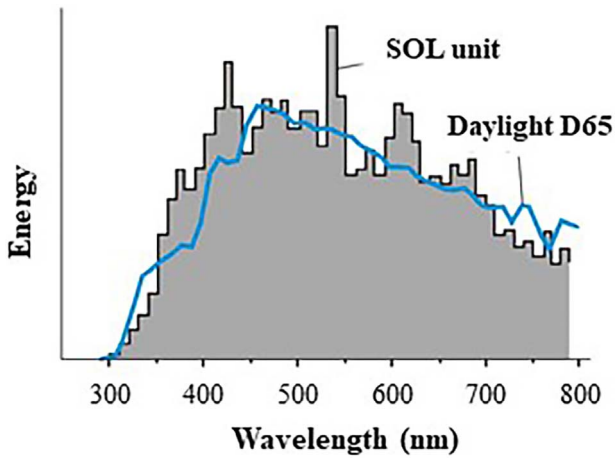

Fig. 1. Spectral distribution of SOL units in comparison with natural sunlight D65. Modified according to producer information (https://www.hoenle.com/products/download-product-information).