

Application of the Validated In Vitro Reconstructed Human Skin and Eye Models For Hazard Identification of the Chemical Mixtures

Kimyasal Karışımların Tehlike Tanımlanması İçin Onaylanmış in Vitro Yapılandırılmış İnsan Deri ve Göz Modellerinin Uygulanması

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ABSTRACT

The international and national directives and legislation requires to perform validated in vitro alternative methods to animal testing of chemicals and mixtures for predictable and precise evaluation of toxicity in human. The regulatory agencies require the hazard assessment on skin and ocular as reported by the United Nations, Globally Harmonized System (UN GHS) of Classification and Labeling of Chemicals. To address this need and investigate the usage of alternative methods, the five representative chemical mixtures were prepared in different compositions and their toxicity was evaluated using in vitro validated EpiDerm™ SCT for skin corrosion, EpiDerm™ SIT for skin irritation, and EpiOcular™ OCL for eye irritation. The tested chemical mixtures 1 and 3 were found corrosive for skin and irritant for both skin and eye. They were identified in the category 1 according to UN GHS. The tested chemical mixtures 2, 4, and 5 were found to be neither corrosive nor irritant to the skin and eye. They were identified in the UN GHS No Category. The potential usage of in vitro Reconstructed Human Epidermis and Cornea-like Epithelial as a replacement to animal testing contributed significantly to classify the ocular and skin irritation/corrosion of liquid and solid chemical mixtures and to comply with the regulatory testing requirements. This study confirm that the use of alternative assays instead of conventional *in vivo* animal test methods could be very reliable and accurate tool to evaluate the skin and eye irritation/corrosion for hazard identification of chemical mixtures.

Keywords: Epiderm, Epiocular, Eye irritation, Hazard identification, Skin irritation

ÖZ

Uluslararası ve ulusal yönetmeliklere ve mevzuata göre, insanda toksisitenin güvenilir ve doğru bir şekilde belirlenmesi için doğrulanmış hayvan deneylerine alternatif in vitro yöntemlerin kullanılması gerekmektedir. Düzenleme kurumları deri ve oküler tehlike değerlendirmesinin Kimyasalların Sınıflandırılması ve Etiketlenmesine İlişkin Birleşmiş Billetler, Global Harmonize Systeme (UN GHS) göre yapılmasını talep etmektedir. Bu ihtiyacı yönelik olarak, hayvan testine alternatif yöntemlerin kullanımını incelemek için beş temsili farklı içeriklerde kimyasal karışım hazırlandı ve toksisiteyi doğrulanmış in vitro EpiDerm™ SCT deri korozyonu, EpiDerm™ SIT deri irritasyonu ve EpiOcular™ OCL göz irritasyonu modelleri kullanılarak belirlendi. Test edilen 1 ve 3 nolu kimyasal karışımlar, deri için korrosive ve deri ve göz her ikisi içinde irritant olarak bulunmuştur. Bu kimyasallar UN GHS'e göre Kategori 1 olarak sınıflandırılmıştır. Test edilen 2, 4 ve 5 nolu kimyasal karışımların deri ve göz için koroziv ve irritant olmadığı bulunmuştur. UN GHS'ye göre sınıflandırılmayan kategorisinde tanımlanmıştır. Yönetmeliklerde yer alan gereksinimlere göre hayvan deneylerine alternatif in vitro yeniden yapılandırılmış insan epidermis ve kornea benzeri epitel modellerin kullanımı, sıvı ve katı kimyasal karışımların deri ve göz irritasyon/korozyon potansiyellerinin sınıflandırılmasına önemli ölçüde katkıda bulunabilir. Bu çalışma, kimyasal karışımların tehlike tanımlanmasında geleneksel in vivo hayvan deneyleri yerine deri ve göz için koroziv/irritant değerlendirilmesinde alternatif deneyler olarak kullanılabileceğini göstermektedir.

Anahtar Kelimeler: Deri irritasyonu, Epiderm, Epiocular, Göz irritasyonu, Tehlike tanımlanması

3Rs Workshop (Replacement, Reduction & Refinement) Workshop on 2D 3D LIVER & SKIN REGULATORY MODELS, 2017, TÜBİTAK MAM

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INTRODUCTION

A wide variety of consumer products such as medical devices, drugs, cosmetics, and chemicals may induce serious skin and eyes irritation or damage as contact with these tissues. Therefore, hazard identification of these products for skin and ocular irritation/corrosion is enforced by the United States (US), European Union (EU) regulatory and Republic of Turkey Ministry of Environment and Urbanization agencies to ensure consumer safety.¹ Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) legislation for these variety of agents and products requires an assessment of the skin and ocular irritation future as stated by the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS).² Although the evolution of skin and eye irritation/corrosion future of chemicals and products has commonly involved the use of animal testing methods more than 40 years, these in vivo tests has been criticized due to their inadequacy of recreatability, misconception of human responses, and the usage of animals.³ These matters have endorsed a number of projects for reduction, refinement and replacement (3Rs) of animal testing. In addition, it was reinforced through the implementation of the 7th Amendment to the Cosmetics Directive prohibited the usage of animals for the safety assessment of cosmetic ingredients and products.⁴ The implementation of 3Rs in Turkey has been started since 2002. Turkish-EU integration attempts have reached dynamism to renovate “Acquis Communautaire” constructed the path to the todays structure. The Turkish Ministry of Agriculture and Rural Affairs disseminated a regulation on the breeding and husbandry of laboratory animals in 2004. All animal facilities have to comformed required technical and operational standards as stated by this directive. Afterwards, in 2006, Turkish Ministry of Environment and Forestry disseminated the regulation on Laboratory Animals Ethics Committees and

settled the “National Ethics Committee for Animal Experiments”.

The European Union Reference Laboratory European Centre for the Validation of Alternative Methods (EURL ECVAM's) assessed the relevance and reliability of the in vitro skin and eye irritation/corrosion assays, which are broadly applicable, straight usage, and economic methods.⁵ The validated three-dimensional (3D) reconstructed human epidermis; EpiSkin™, SkinEthic™ RHE, epiCS®, EpiDerm™, and LabCyte EPI-MODEL24 are mimics the morphological, and biochemical characteristics of the outhar layer of the human skin. They provides the evaluation of skin irritation/corrosion futures of chemicals and mixtures according to the UN GHS.²

The two validated 3D RhCE tissues are reconstructed from primary human transfected corneal epithelial cells (SkinEthic™ HCE/S) and from human epidermal keratinocytes (EpiOcular™ OCL-200) measure cytotoxicity occurred throughout the production of cell and tissue damage in the cornea due to penetration of the chemicals.^{6,7} To replace the in vivo animal testing, many alternative tissue models have been developed but several of them have been validated and officially approved as test guidelines (TGs) via the Organization for Economic Cooperation and Development (OECD).⁸

The aim of this study to explore the application of validated in vitro 3D human reconstructed models of eye and skin for the hazard identification of the five representative chemical mixtures. The validated EpiDerm™ SCT, EpiDerm™ SIT and EpiOcular™ OCL models were selected as the in vitro testing models because of their broad relevancy to different types of chemicals, medical devices, cosmetics, and mixtures. The increased usage of validated in vitro systems as animal alternatives may explore their development and validation studies.

MATERIALS AND METHODS

This study supported by Marmara Research Center Foundation with the project number of 57A3101.

In Vitro Skin Corrosion

In vitro EpiDerm™ SCT reconstructed from human derived epidermal keratinocytes, RhE was purchased from MatTek Corporation, Bratislava, Slovakia (Figure 1). Following the application of the chemicals mixtures in the Table 1, the occurrence of irreversible adverse effects to the skin revealed as noticeable tissue death on the epidermis and into the dermis indicated as skin corrosion. This test allowed the classification of non-corrosive and corrosive chemical mixtures. The assay was carried out as guided by the OECD TG 431 and the details of the method was given in the Table 2.⁹

Table 1. The Compositions of the Chemical Mixtures Tested by Using in Vitro Reconstructed Skin and Eye Models.

Chemical mixtures, CM	%*
Chemical mixture 1 (CM 1), solid	
Polycarbonate (p#** GF65553598)	10.7
Potassium nitrate (p# 221295)	64.3
Ironoxide (p# GF37175665)	7.1
Magnesium carbonate	17.9
Hydroxypentahydrate (p# M5671)	
Chemical mixture 2 (CM 2), solid	
Terephthalic acid (p# 490849)	35.0
Potassium chlorate (p# 255572)	28.0
Sucrose (p# 94474)	17.0
Magnesium carbonate	20.0
Hydroxypentahydrate	
Chemical mixture 3 (CM 3), solid	
2,4-Dichlorobenzylidene-Malono nitrile (p# S531715)	35.0
Potassium chlorate	25.0
Sucrose	25.0
Hydromagnesite (p# 1.05827)	15.0
Chemical mixture 4 (CM 4), liquid	
Oleoresin capsicum (p# M2028)	0.3
Propylene glycol (p# W294004)	39.7
Isopropyl alcohol (p# W292907)	60.0
Chemical mixture 5 (CM 5), liquid	
Capsaisin	0.2
Propylene glycol	38.8
Isopropyl alcohol	60.0

*%: Percentage in the chemical mixture

**p#: Product number

Briefly, the liquid or solid tested chemical mixtures was prepared and added to the upper sides of the tissue insert (0.63 cm²) in the 24-well plate. It was incubated either 3 min at 25 °C or 60 min exposure time at 37 °C, 5% CO₂. Following the treatments, the inserts were washed with Dulbecco's Phosphate Buffered Saline (DPBS, p# 8537) several times. The cell viability was investigated by adding 300 µl of medium including 10% of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (p# M5655, SigmaAldrich) dissolved in DPBS (1 mg/ml). The mitochondrial enzymes of the viable cells can convert the yellow MTT into a blue formazan salt. After 3 hrs dissolving of formazan salt from the tissues with dimethyl sulfoxide (p# D8418), It was quantitatively measured at 570 nm with microplate reader.

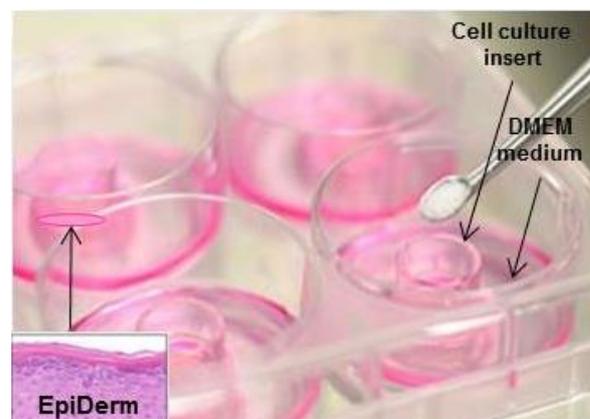


Figure 1. In vitro EpiDerm™ reconstructed from human derived epidermal keratinocytes, RhE (MatTek Corporation, Bratislava, Slovakia).⁹

In Vitro Skin Irritation

The prepared chemical mixtures which were given in the Table 1 placed topically to the EpiDerm™ SIT tissues consisting human derived non-transformed and highly differentiated epidermal keratinocytes was purchased from MatTek Corporation, Bratislava, Slovakia. It mimics the in vivo main intercellular lamellar lipid layers composed of a multilayered stratum corneum. In vitro skin irritation using EpiDerm™ SIT was carried out as described by OECD TG 439 and the details of the

method was given in the Table 2.¹⁰ After treatment and post-treatment incubation period of the tissues, if the tissue viability was \leq to 50%, the tested chemical mixtures were classified as Category 2 or 1 as defined by the UN GHS.

In Vitro Eye Irritation

The prepared chemical mixtures given in the Table 1 were applied topically to the EpiOcular™ corneal model (OCL-200, MatTek Corporation, Ashland, MA). It is derived from the human epidermal keratinocytes which mimics the form a stratified, squamous epithelium found in the cornea. The assay was carried out as defined by the OECD TG 492 and the details of the method was given in the Table 2.¹¹ The method was applied to assess the eye hazard characteristics of the tested chemical mixtures based on their adverse effects on the in vitro validated EpiOcular cornea model, measured by the tetrazolium dye. The viability of the tissues following exposure to the tested chemical mixtures was analyzed in comparison with the tissues treated with the negative control substance which was set to 100% viability.

Aspect of Research Ethics

In this study, an ethics committee application is not required, as the study was conducted on commercial in vitro models.

This research has been conducted in the laboratory of TUBITAK, Marmara Research Center, Genetic Engineering and Biotechnology Institute. The institutional permission for this study was received on October 28, 2020.

Data Analysis

The data of the mean values and the standard deviations of the 3 replicates was calculated with Microsoft Excel 2016 and presented as mean \pm Stdev. The graphs for each in vitro models were drawn with the Microsoft Excel program. The significance for each method was considered according to the standards given in the each of the applied methods.

Limitation of Research

This study was carried out using validated in vitro models which could not be easily available in Turkey. These models needs to be imported from abroad. In addition, they have very short shelf life for their usage.

Conflict of Interest

There was not any conflict of interest which was declared by the author.

Financial Disclosure

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Table 2. Test Methods of the in vitro Validated Epiderm™ SCT for Skin Corrosion, Epiderm™ SIT for Skin Irritation, and Epiocular™ OCL for Eye Irritation.

Test Method Components	EpiDerm SCT	EpiDerm SIT	EpiOcular OCL
Model surface	0.63 cm ²	0.60 cm ²	0.60 cm ²
Number of tissue replicates	n = 3 tissues used for each chemical mixture	n = 3 tissues used for each chemical mixture	n = 3 tissues used for each chemical mixture
Exposure doses and treatment	Liquids: 50 μ l or 79.4 μ l/cm ² Solids: 25 μ l H ₂ O + 25 mg (39.7 mg/cm ²)	Liquids: 50 μ l or 83.3 μ l/cm ² Solids: 50 mg or 83.3 mg/cm ²	Liquids: 50 μ l or 83.3 μ l/cm ² Solids: 50 mg or 83.3 mg/cm ²
Exposure time and temperature	3 min at 25°C, and 60 min at 37°C, 5% CO ₂	Liquids: 30 min Solids: 6 hrs at 37°C, 5% CO ₂ in culture medium	Liquids: 30 min Solids: 6 hrs at 37°C, 5% CO ₂ in culture medium
Rinsing	20 times with DPBS*	3 times in 100 ml of Ca ²⁺ /Mg ²⁺ free DPBS	3 times in 100 ml of Ca ²⁺ /Mg ²⁺ free DPBS
Post-exposure preoccupation	---	Liquids: 12 min Solids: 25 hrs at RT** in culture medium	Liquids: 12 min Solids: 25 hrs at RT in culture medium

Table 2 continue

Post-exposure incubation	---	Liquids: 120 min Solids: 18 hrs at 37°C, 5% CO ₂ in culture medium	Liquids: 120 min Solids: 18 hrs at 37°C, 5% CO ₂ in culture medium
Negative control (NC)	50 µl H ₂ O	50 µl H ₂ O	50 µl H ₂ O
Positive control (PC)	50 µl 8N KOH	50 µl Methyl acetate	50 µl Methyl acetate
MTT solution	300 µl (1 mg/ml)	300 µl (1 mg/ml)	300 µl (1 mg/ml)
MTT incubation time & temperature	180 min at 37°C, 5% CO ₂	180 min at 37°C, 5% CO ₂	180 min at 37°C, 5% CO ₂
Extraction solvent	Isopropanol, 2 ml	Isopropanol, 2 ml	Isopropanol, 2 ml
Extraction time and temperature	mixing at 120 rpm at 25°C for 2-3 hrs	mixing at 120 rpm at 25°C for 2-3 hrs	mixing at 120 rpm at 25°C for 2-3 hrs
OD reading	570 nm	570 nm	570 nm
Tissue Quality Control	Using Triton X-100 in 100 µl of 0.3% (v/v) 4 hrs ≤ ET ₅₀ *** ≤ 8.7 hrs	Using Triton X-100 in 100 µl of 0.3% (v/v) 12.2 min ≤ ET ₅₀ ≤ 37.5 min	Using Triton X-100 in 100 µl of 0.3% (v/v) 12.2 min ≤ ET ₅₀ ≤ 37.5 min
Acceptability Criteria	Optical density of the tissues exposed to the negative control should be ≥ 0.8 and ≤ 2.8. Percent viability of the tissues exposed to the positive control for 1 hr defined as % of the negative control should be ≤ 15%	Optical density of the tissues exposed to the negative control should be > 0.8 and < 2.5. Percent viability of the tissues exposed to the positive control for 6 hrs defined as % of the negative control should be < 50%	Optical density of the tissues exposed to the negative control should be > 0.8 and < 2.5 Percent viability of the tissues exposed to the positive control (30 min for liquids and 6 hrs for solids) defined as % of the negative control, should be < 50%

*DPBS: Dulbecco's Phosphate Buffered Saline; **RT: Room temperature; ***ET₅₀: The exposure time required to reduce cell viability by 50%

RESULTS AND DISCUSSION

In Vitro Skin Corrosion

The occurrence of irreversible adverse effects to the skin revealed as noticeable tissue death over the epidermis and into the dermis is defined as skin corrosion following exposure to the chemicals and the other products. RhE Test Method using EpiDerm™ SCT contributes the classification of non-corrosive, corrosive, and also partial sub-categorisation in accordance with UN GHS.¹² When CM 1 and CM 3 was applied to the in vitro EpiDerm tissues, the tissue viability was resulted in < 50% and < 15% after exposure for 3 min and 60 min, respectively. Therefore, CM 1 and CM 2 were corrosive and placed in the category 1A. The tissue viability upon exposure to CM 2, CM 4, and CM 5 for 3

min and 60 min were found ≥ 50% and ≥ 15%, respectively. The corrosive and non-corrosive compounds were shown in the

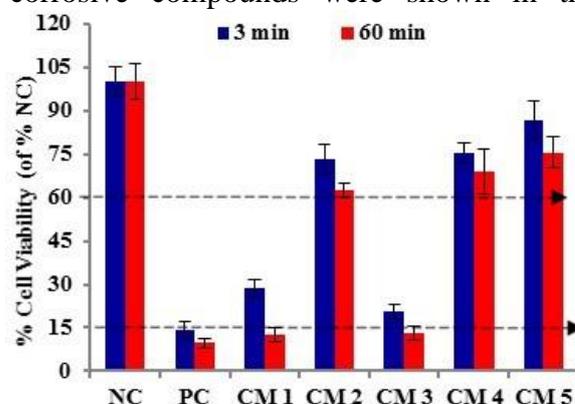


Figure 2.

Figure 2. In Vitro Skin Corrosion Test Results Obtained by Using EpiDerm™ Tissues (n = 3) for 5 Chemical Mixtures (CM), NC and PC.¹²

The *in vitro* skin corrosion, RhE Method contributes significantly in analyzing the skin corrosion future of a wide range of liquid and solid chemical mixtures as stated by the UN GHS. Deprez et al. (2015) confirmed the predictive potential of EpiDerm tissue model by testing corrosivity potential of the 80 chemicals comparing the results with the other *in vitro* tissue models such as EpiSkin™ SkinEthic™ and epiCS®.¹² In addition, tis model allows allowtranslating those values into sub-categories of corrosive chemicals.

In Vitro Skin Irritation

It is based on the *in vitro* test model of RhE which represents the upper layer of the human skin and their biochemical and physiological properties. The mean percent of the tissue viability after exposure to the tested CM1, and CM 3 was less than (\leq) 50%. Therefore, the tested CM 1, and CM 3 were identified as skin irritants in the category 2 according to UN GHS. The tissue viability after exposure to the tested CM 2, CM 4, and CM5 was found more than ($>$) 50% as compared with the negative control (H₂O). In accordance with UN GHS, these mixtures were classified in the no-category as non-irritants to the skin and identified in the no Category (Figure 3).

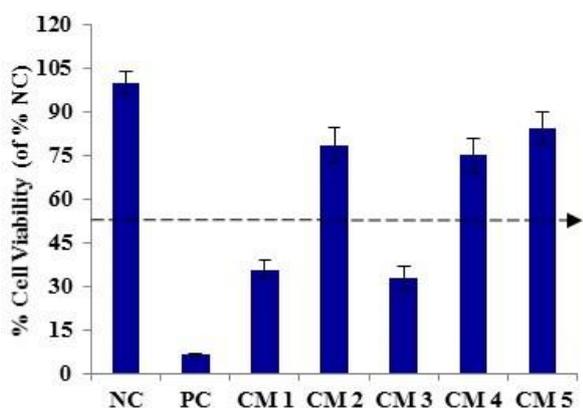


Figure 3. In Vitro Skin Irritation Results Obtained by Using EpiDerm™ Tissues (n = 3) for 5 Chemical Mixtures (CM), NC and PC.¹³

In the another study, the capacity of EpiDerm tissue model to address the skin irritation potential of medical devices was also demonstrated comparing with conventional

Draize rabbit skin irritation test.¹³ The result of this study confirmed our findings.

In Vitro Eye Irritation

The serious eye damage may occurs upon treatment of the eye to a test chemical due to severe physical impairment of vision. If it is not fully reversible, the chemical is defined in the UN GHS Category 1. On the other hand, if the induction of eye irritation are fully reversible, these chemicals and mixtures are determined as UN GHS Category 2. When the test chemicals are not classified for serious eye damage eye or irritation, they are assigned to as UN GHS No Category.

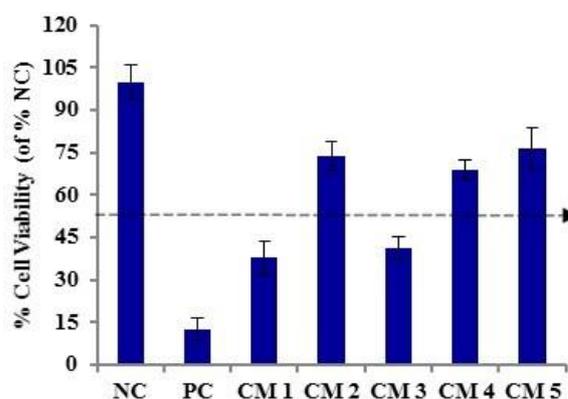


Figure 4. In Vitro Eye Irritation Results Obtained by Using EpiOcular™ Tissues (n = 3) for 5 Chemical Mixtures (CM), NC and PC.¹³

The mean percent tissue viability after exposure to the tested CM1 and CM 3 was less than (\leq) 50%. They were eye irritants and identified as category 1 according to UN GHS. The tissue viability after exposure to the tested CM 2 CM 4 and CM5 was more than ($>$) 50%. These mixtures were considered as non-irritant to the eyes as defined by the UN GHS No Category (Figure 4). In another study, the eye irritation potential of 20 nanosized materials using *in vitro* EpiOcular tissue model was assessed.¹⁴ The findings showed that the model allowed to determine the irritation potential of these materials as obtained from our study. EpiOcular tissue model was also used to evaluate eye irritation potential of medical devices.¹⁵ The results showed that this model can be replaced the conventional *in vivo* test for medical devices.

CONCLUSION

This study was carried out to explore the use of validated in vitro tissue models as animal testing alternatives. In addition, these in vitro models are generally used for single chemical/product whereas they are rarely used for mixtures for hazard identification. This study was also confirmed the usefulness of these in vitro models for chemical mixtures.

The implementation of 3Rs in Turkey, in the process of grant application to TÜBİTAK which has required ethics committee approval from the researchers, Turkish- EU integration attempts have increased dynamism and the restructure to integrate “Acquis Communautaire” builded since 2002. The Ministry of Agriculture and Rural Affairs disseminated a regulation on the breeding and husbandry of laboratory animals in 2004. According to this regulation, all animal

facilities have to supply required technical and operational needs. Consequently, in the aspects the implementation of the 3R principles, the Ministry of Environment and Forestry declared the regulation on Laboratory Animals Ethics Committees and established the “National Ethics Committee for Animal Experiments” in 2006.

There is a need to develop and validate in vitro models for testing consumer products such as medical devices, drugs, cosmetics, and a number of chemicals and mixtures that may cause severe acute and chronic damage in the variety organs and in the environment. In addition, it is very important to collabrate with the international research centers in the process of validation of the in vitro organ models due to their very crucial usage at the research base and also at the regulatory requirements stage.

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