JAGIELLONIAN CENTER OF INNOVATION

APPLICATION NOTE – JAGIELLONIAN CENTER OF INNOVATION

IN-VITRO STUDY OF THE CYTOTOXICITY OF COSMETIC SUBSTANCES

Katarzyna Pocheć*, Natalia Wojtyniak*,**

*Laboratory of Molecular Biology. Jagiellonian Center of Innovation, ul. Bobrzyńskiego 14, 30-348 Kraków

**Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-001 Kraków

ABSTRACT

A ban on animal testing of cosmetics introduced in 2009 in the European Union is pushing cosmetics producers to use different methods for guaranteeing safety to their consumers. One available, and already validated, method is the SkinEthic (RHE) Skin Irritation Test.

INTRODUCTION

Skin irritation is considered to be a reversible form of damage to the skin triggered by the application of a test chemical for up to 4 hours[1]. Categorizing cosmetic chemicals as either irritant or non-irritant was typically based on animal in-vivo testing. However, experimenting on animals fails to predict the outcome of the same procedure or application of the same substance in humans and thus can pose a danger for human health[2].

Animal testing of cosmetics or their ingredients has been banned in the EU since 2009, according to the Regulation (EC) No. 1223/2009 and others. The purpose of the ban is to put an end to animal cruelty as well as to develop more reliable methods of classifying chemicals that can come in contact with the human body. EURL ECVAM (European Union Reference Laboratory for alternatives to animal testing of the European Centre for the Validation of Alternative Methods) is an organization responsible for proposing and validating non-animal testing methods. One of the methods that has been validated for classifying cosmetic ingredients as skin-irritating is the SkinEthic RHE Skin Irritation Test [3].

The test is designed to predict and classify the acute skin irritation potential of chemicals by measuring their cytotoxic effects, as reflected in the MTT assay on the Reconstructed Human Epidermis (RHE) model. The protocol is compliant with the OECD Test Guideline No. 439 – In Vitro Skin Irritation[4].

The SkinEthic™ RHE tissue model consists of normal human keratinocytes and presents a histological morphology comparable to the in vivo human epidermis, comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum[4].

The basis of the test consists of using 19-day-grown reconstructed human epidermis (RHE) inserts, cultured on a 0.5 cm2 insert polycarbonate filter that comes in contact with a growth medium from the basal side of the epidermis and the air from the apical side, and then topically exposed to the tested substance, which can be a liquid, a pulverized solid, a semi-liquid or even a wax[4,5].

The liquids may be aqueous or non-aqueous, and solids may be soluble or insoluble in water[5]. After that the epidermis inserts are washed and undergo a long post-treatment incubation period, which allows the clear cytotoxic effects to appear [4]. At the end, an MTT assay is conducted to assess the cytotoxicity of the tested substance. If the mitochondrial dehydrogenases reduction of MTT[5] is more than 50% compared to negative control, the substance is considered non-irritant; if it is less than 50%, it is irritant[5].

The aim of carrying out the procedure was to demonstrate the technical proficiency of the lab staff in applying the skin irritation test.

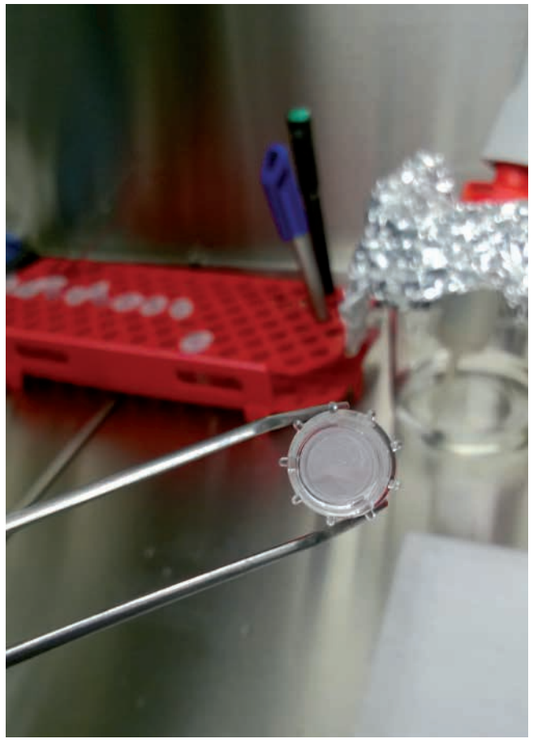

Figure 1. *Single tissue insert*

RESULTS AND CONCLUSIONS:

The reduction of cell viability in treated tissues is compared to treated tissues with negative control (100% viability) and expressed as a percentage.

Substance	Cell viablity	Results
Naphthalene acetic acid	113.14%	Non-irritant
Isopropanol	110.72%	Non-irritant
Methyl stearate	108.54%	Non-irritant
Heptyl butyrate	120.83%	Non-irritant
Hexyl salicylate	82.17%	Non-irritant
Cyclamen aldehyde	2.89%	Irritant
1-bromohexane	3.01%	Irritant
Potassium hydroxide (5% aq.)	1.03%	Irritant
1-methyl-3-phenyl-1-piperazine	53.41%	Irritant
Heptanal	1.98%	Irritant

Table 1. *Cell viability results*

The obtained results are in compliance with the guideline data. Five of the compounds showed a non-irritant effect on the epidermis, while the other five had a strong irritant influence on the tissue.

The result of one of the substances (1-methyl-3-phenyl-1-piperazine) turned out to be on the borderline. However, considering the fact that it is a solid, the results may have been the effect of unequal substance spreading on the tissue during application.

MATERIAL AND METHODS

PROFICIENCY SUBSTANCES

Proficiency substances are a subset of the substances, which are commercially available; they represent the full range irritancy scores (from non-irritant to strong irritant); they have a well-defined chemical structure; they provided reproducible in vitro results across multiple testing and multiple laboratories; they were correctly predicted in vitro, and they are not associated with an extremely toxic profile (e.g. carcinogenic or toxic to the reproductive system)[4].

According to the recommendation, 10 substances of a known irritation status were used: naphthalene acetic acid, isopropanol, methyl stearate, heptyl butyrate, hexyl salicylate, cyclamen aldehyde, 1-bromohexane, potassium hydroxide (5% aq.), 1-methyl-3-phenyl-1-piperazine and heptanal. Among these 10, the first five are considered non-irritant (two are optional mild irritants, but under this Test Guideline, optional mild irritants are categorized as non-irritant) and the last five are known to be typically irritant to the human skin [5].

Substance	CAS NR	Physical state	UN GHS Category	Results
Naphthalene acetic acid	86-87-3	Solid	No Cat.	Non-irritant
Isopropanol	67-63-0	Liquid	No Cat.	Non-irritant
Methyl stearate	112-61-8	Solid	No Cat.	Non-irritant
Heptyl butyrate	5870-93-9	Liquid	No Cat. (Optional Cat. 3)	Non-irritant
Hexyl salicylate	6259-76-3	Liquid	No Cat. (Optional Cat. 3)	Non-irritant
Cyclamen aldehyde	103-95-7	Liquid	Cat. 2	Irritant
1-bromo-hexane	111-25-1	Liquid	Cat. 2	Irritant
Potassium hydroxide (5% aq.)	1310-58-3	Liquid	Cat. 2	Irritant
1-methyl-3-phenyl-1 -piperazine	5271-27-2	Solid	Cat. 2	Irritant
Heptanal	111-71-7	Liquid	Cat. 2	Irritant

Table 2. *Proficiency substances*

TISSUE TREATMENT

After receipt, the tissue is transferred to a Growth Medium and pre-incubated in a humidified incubator at 37°C under a 5% CO_2 and 95% air atmosphere overnight in a 6-well plate or at least for 2 hours in a 24-well plate.

Then the inserts are transferred to a Maintenance Medium in a 24-well plate and the tested substances are topically applied on the tissue: 16 µL+/-2 µL for liquids or viscous and 16 mg+/-2 mg for solids, respectively. Each chemical should be repeated on 3 tissues, and then the epidermis is exposed to the tested substance for 42 minutes at room temperature.

The negative control is PBS and the positive control is 5% SDS, both tested in triplicate as well.

After this, the epidermis inserts are washed and incubated at 37°C under a 5% CO_2 and 95% air atmosphere in a Growth Medium in a 6-well plate for 42 hours.

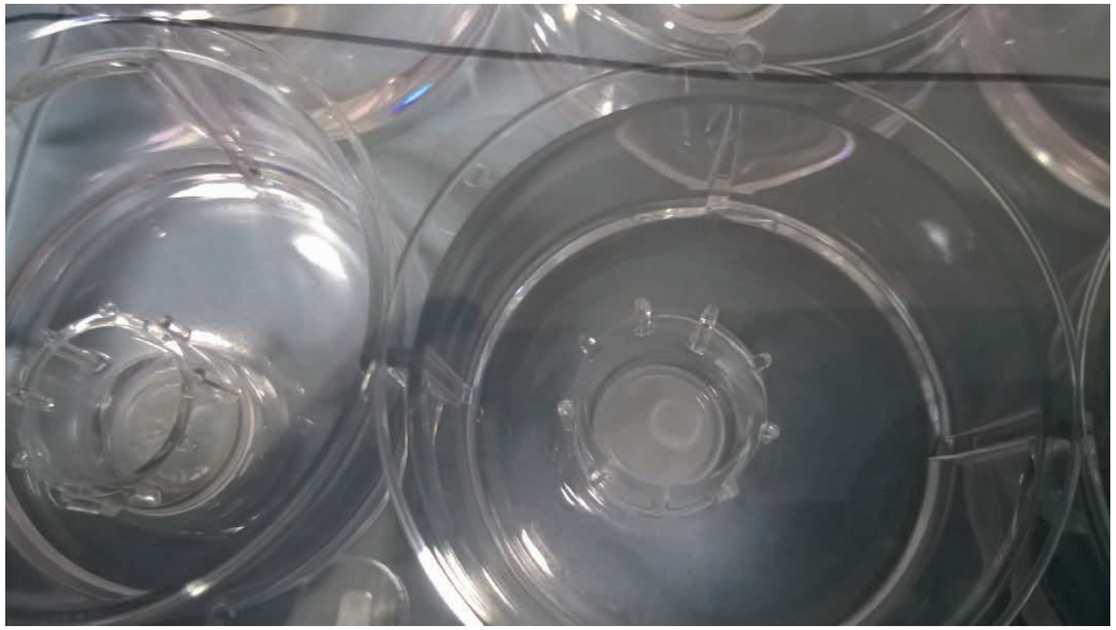

Figure 2. *Epidermis inserts in a Growth Medium in a 6-well plate*

CELL VIABILITY

To evaluate the cytotoxicity of the tested substance, an MTT assay is conducted. The tissue is transferred into a 1 mg/ml MTT solution in a 24-well plate and incubated for 3 hours at 37°C under a 5% CO_2 and 95% air atmosphere. Then the inserts are immersed in isopropanol and left for at least 2 hours at room temperature, protected from light, on a plate shaker for formazan to be extracted.

The amount of the formazan is quantified by measuring the optical density (OD) at 570 nm with a plate reader (Tecan Spark 10M).

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CONTACT

Łukasz Kutrzeba, PhD
Director Business Development

K: +48 515 075 500
E: lukasz.kutrzeba@jci.pl

Jagiellonian Center of Innovation
ul. Bobrzyńskiego 14, 30-348 Kraków

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