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IN VITRO EFFICACY STUDY
EVALUATION OF THE EFFICACY OF A COSMETIC PRODUCT IN PROTECTING THE SKIN AGAINST DAMAGE AGENTS

RIVOLI COSMÉTIQUES SA

L'EAU DE JOUR N'02

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Capitale Sociale € 161.834 i.v. C.C.I.A.A. MI 1437929 - Registro Società N. 342215
Centro di Saggio operante in BPL - Sistema di gestione qualità certificato ISO 9001
Iscritto al Registro Regione Lombardia ai fini dell'autocontrollo alimentare (N 030015309008)

INDEX

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REPORT CHANGE RECORD

The table here below reports the change log of all approved changes made to the document that make up the course after initial approval.

Rev. no	Date	Description
00	06/10/2022	Draft report release
00	19/10/2022	Report release

1. STUDY DESIGN

1.1 Title

In vitro efficacy study – evaluation of the efficacy of a cosmetic product in protecting the skin against damage agents.

1.2 Study aim

The study described below aimed to evaluate the protecting efficacy of a cosmetic product in a model of human reconstructed in vitro epidermis exposed to a series of damaging agents (ozone, blue light and UV rays).
For this way the capability of the product subjected to the test to protect the reconstructed tissue after exposure to the damage agents was evaluated by determination of viability, of cellular metabolism, of level of lipid lipoperoxidation by MDA assay and of DNA damage.
In summary, the reconstructed tissues are treated with the product and then damaged by repeated exposure to ozonized medium, blue light and UV radiation by solar simulator.
At the end of the treatment, cell viability is measured, and media and homogenates are collected to carry out the dosages.
Results are compared with untreated tissues (CTR-) and untreated tissues and exposed to damage agents (CTR+).

1.3 Tested product

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Sample description

Cosmetic product with the following INCI composition declared by Sponsor:

Name (INCI) GLOB	Content [%]
AQUA (WATER)	**85.98130**
PROPANEDIOL	**5.00000**
NIACINAMIDE	**3.00000**
1,2-HEXANEDIOL	**2.50000**
GLYCERIN	**1.52800**
PROPYLENE GLYCOL	0.80000
CAPRYLOYL/CAPROYL ANHYDRO METHYL GLUCAMIDE	0.49000
PARFUM (FRAGRANCE)	0.20000
FUMARIC ACID	0.12500
POLYDATIN GLUCOSIDE	0.10000
CITRIC ACID	0.08350
SODIUM CITRATE	0.07500
METHYLGLUCOSIDE PHOSPHATE	0.05000
COPPER LYSINATE/PROLINATE	0.03000
CITRUS LIMON (LEMON) FRUIT EXTRACT	0.01250
FUMARIA OFFICINALIS FLOWER/LEAF/STEM EXTRACT	0.01250
DISODIUM ACETYL GLUCOSAMINE PHOSPHATE	0.01200
CI 14700 (RED 4)	0.00020
Total:	**100.00000**

1.4 Dates of test execution

Tissue treatment: 21/09/2022 – 23/09/2022
Dosaggi / Dosages: 26/09/2022
Analisi dati/ Data analysis: 28/09/2022

2. MATERIALS AND METHODS

2.1 Experimental model

The biological model used in the test consists of three-dimensional reconstructed human epidermis, built from primary cultures of keratinocytes (EpiDerm – MatTek, batch 36172). Particularly, this is 0.6 cm^2 reconstructed epidermis by airlifted culture of keratinocytes for 17 days in chemically defined medium on inert polycarbonate filter at the air/liquid interface. The test system has an ultra-structure (tissue morphology and thickness) very similar to the human in vivo skin. All the tissue units were subjected to quality controls to ensure the suitability of the biological model and verify the absence of pathologies to ensure the operator safety.

EpiDerm – MatTek, batch 36172

2.2 Preparation of the test item and tissue exposure

For the test execution, reconstructed human epidermis were first treated with product under study and then exposed to the following damage agents for a total time of 72h:

- UV;
- Blue light;
- Ozone.

In order to evaluate the protective capability of test item against each one of selected damage agents, the treated tissues were subjected to the following experimental conditions:
- 2 hours/day repeated exposure to UV irradiation by means of a solar simulator;
- 8 hours/day repeated exposure to blue light diffused by LED lamps;
- 1 hour/day repeated exposure to ozone, by means of tissue contact with ozonized medium.

At the end, the viability of the tissues was determined by MTT assay, the underlying culture media and the tissue homogenates were collected for the determination of protein synthesis, lipoperoxidation level by malondialdehyde assay and evaluation of DNA damage by means of 8-OHdG dosage. The results were compared with the negative control (untreated epidermis, CTR-) and with the positive control (tissues untreated, but exposed to each one of the three damaging agents, CTR +s).

In summary, the experimental protocol provided:

- untreated epidermis (negative control, CTR-);
- untreated epidermis, but exposed to one of the three damaging agents (positive controls, CTR+);
- epidermis treated with the test item and then exposed to each one of the three damaging agents.

2.3 Cell viability study – MTT assay

The MTT test (3,(4,5-dimethylthiazol-2)2,5 difeniltetrazolium bromide) is a standard, simple and accurate colorimetric method for cell viability assessment. The assay is based on the intracellular reduction of the yellow tetrazolium salts by the mitochondrial enzyme succinate dehydrogenase in blue/purple formazan crystals. The reaction may therefore take place only in metabolically active cells and the value of the optical density obtained by spectrophotometric reading can be correlated to the amount of viable cells.
At the end of each treatment the tissues were rinsed with PBS, stained with MTT solution 1 mg/mL and incubated for

three hours at 37°C/5% CO_2. Then the tissues were treated with isopropanol and incubated for two hours at room temperature. After incubation absorbance readings was performed at 570 nm by microplate reader (isopropanol was used as blank for reading). For each test condition the ratio of the average optical density of the treated tissues on the average optical density of negative controls determines the viability rate. The difference between the viability of the sample and the positive control is calculated, direct index of the protective capacity of the product.

2.4 Lipid damage study – MDA dosage

Malondialdehyde (MDA) content determination was used as oxidative stress index linked to the lipid component. The malondialdehyde is in fact a specific biomarker of oxidative stress for lipids reflecting the state of lipid peroxidation. This phenomenon begins at the level of polyunsaturated fatty acids of the membrane phospholipids; free radicals react with phospholipids by oxidizing them and thus lead to the formation of unstable lipid hydroperoxides that decompose producing a number of secondary products such as aldehydes and ketones recognized as toxic or carcinogenic substances. After the beginning and propagation of lipid peroxidation in biological membranes, the products may in turn trigger new radical reactions resulting in a harmful biological ripple effect. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) are the two main products of lipid peroxidation, and their concentration in biological systems is a good index of lipo-peroxide damage.
To determine the lipoperoxides levels the colorimetric method by Erdelmeier and collaborators (1998) was assayed: the test is based on the capability of a chromogen, N methyl 2 phenylindole (NMPI), to react with MDA at 45°C with acid pH to produce a stable blue chromophore that has an absorption pick between 540 and 590 nm.
The quantitative determination uses a calibration curve made-up of known and growing concentrations of standard MDA. The results are expressed as MDA concentration (µM) in 100 µL tissue homogenate.
The difference in % variation of MDA content between positive control and sample vs negative control is calculated, direct index of the protective capacity of the product.

2.5 Cell metabolism study – Protein dosage

Protein content determination was carried out by Lowry et al (1951) colorimetric method. The assay based on the principle that in alkaline condition Cu+ ions make complex with proteins and catalyze the oxidation of tyrosine and tryptophan residues. This oxidation causes the reduction of Folin-Ciocalteau reactive, changing its color from yellow to blue. The colour intensity is proportional to the protein content. The results are expressed as % increase vs a control condition (CTR).

2.6 Study of DNA oxidative damage by 8OHdG analysis

At the end of the treatment, the cell cultures were processed for the DNA purification on which the dosage of 8-OHdG marker was performed.
Commercially available ELISA kit is used. ELISA kits use the competitive binding between an antigen and its primary antibody. The immune complex (antigen-antibody) was bond by a secondary antibody conjugated to a peroxidase. The addition of the enzyme substrate gives a colorimetric reaction with intensity proportional to the immune complex presence, and so to the target molecule.
The quantitative determination uses a calibration curve made-up of standard known and growing concentrations of standard marker.

2.7 Statistical analysis

Obtained results were subjected to statistical analysis by means of Student test. The variations versus negative control and positive control are considered significant for $p<0.05$.

2.8 Bibliographic references

Establishment of a blue light damage model of human retinal pigment epithelial cells in vitro. Su G, Cai SJ, Gong X, Wang LL, Li HH, Wang LM. Genet Mol Res. 2016 Jun 24.

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3. RESULTS

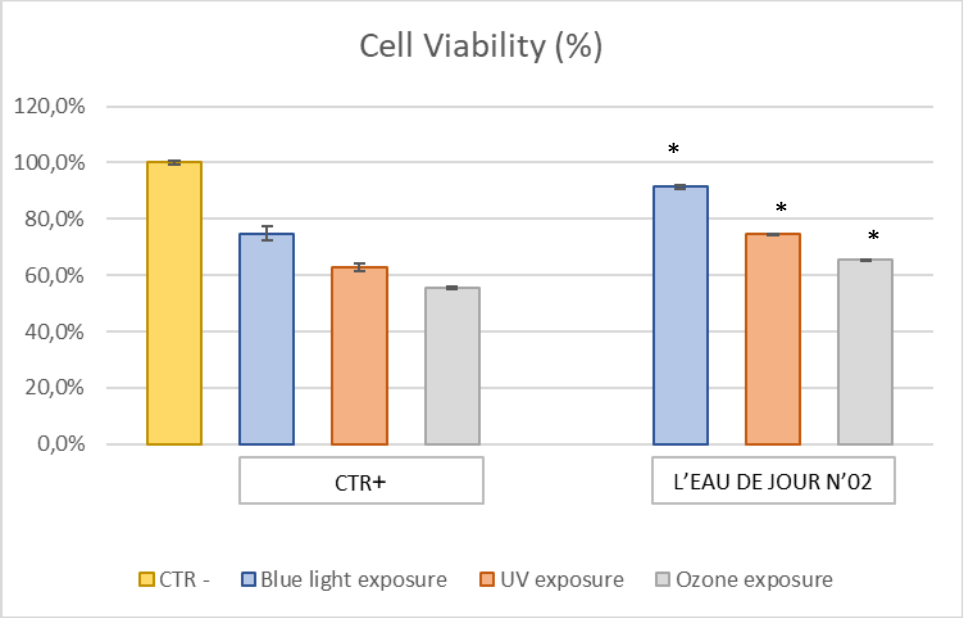
VIABILITY

Table and graph 1

Cell viability in CTR-, CTR+ and epidermis treated with tested product. The results are expressed as mean ± st. dev. (expressed in %) and % protection compared to CTR+.

Protection vs Damaging agent - Cell Viability (%)

	Mean	St. Dev.	VAR% vs CTR-	% Protection	
CTR -	100,0%	0,6%			
Blue light exposure					
CTR+	74,8%	2,5%	-25,2%		
L'EAU DE JOUR N'02	91,3%	0,9%	-8,7%	16,53%	*
UV exposure					
CTR+	62,8%	1,4%	-37,2%		
L'EAU DE JOUR N'02	74,7%	0,2%	-25,3%	11,86%	*
Ozone exposure					
CTR+	55,5%	0,4%	-44,5%		
L'EAU DE JOUR N'02	65,6%	0,1%	-34,4%	10,16%	*



The tissues exposed to damage agents (CTR+) showed a significant reduction of the cell viability ($p<0.05$). Tissues treated with the test product showed a statistically significant protection in cell viability against the damages induced by the blue light, UV irradiation and ozone exposure, compared to the positive control (*$p<0.05$).

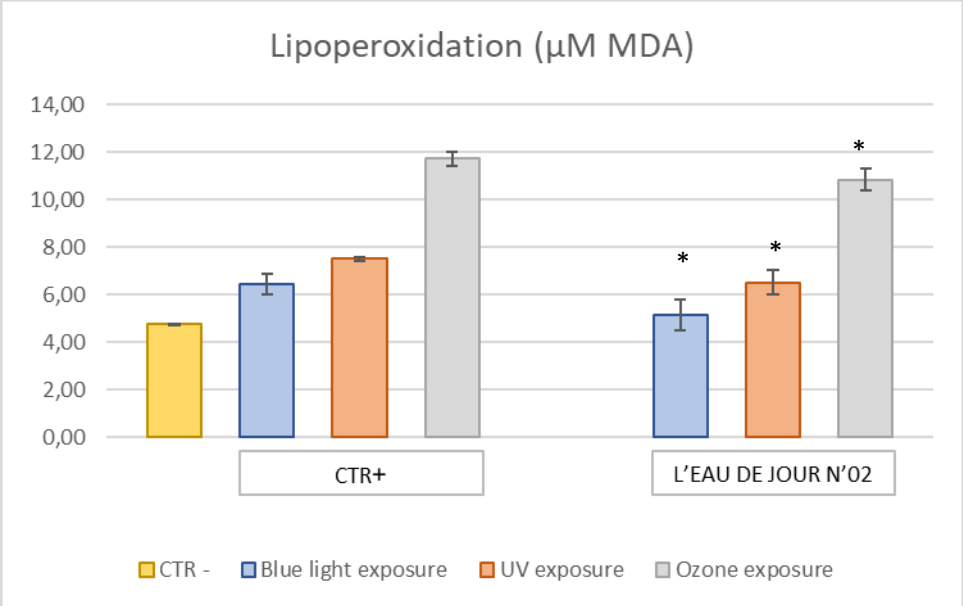
LIPID PEROXIDATION DAMAGE

Table and graph 2

MDA dosage in CTR-, CTR+ and epidermis treated with the tested product. The results are expressed as mean ± st. dev. (uM MDA), mean % variation compared to CTR- and % protection compared to CTR+.

Protection vs Damaging agent - Lipoperoxidation (µM MDA)

	Mean	St. Dev.	VAR% vs CTR-	% Protection	
CTR -	4,76	0,23			
Blue light exposure					
CTR+	6,45	0,43	35,6%		
L'EAU DE JOUR N'02	5,12	0,65	7,6%	27,96%	*
UV exposure					
CTR+	7,50	0,10	57,6%		
L'EAU DE JOUR	6,50	0,51	36,6%	21,06%	*
Ozone exposure					
CTR+	11,74	0,30	146,7%		
L'EAU DE JOUR N'02	10,85	0,45	128,1%	18,64%	*



The tissue exposure to damage agents showed a significant increase of the oxidative damage ($p<0.05$).
The tissues pretreated with the test item and then exposed to damage agents showed MDA levels significantly lower than the positive control (*$p<0.05$).
Moreover the level of LPOin the cells treated with tested product and exposed to blue light are similar to the level of LPO of CTR-.

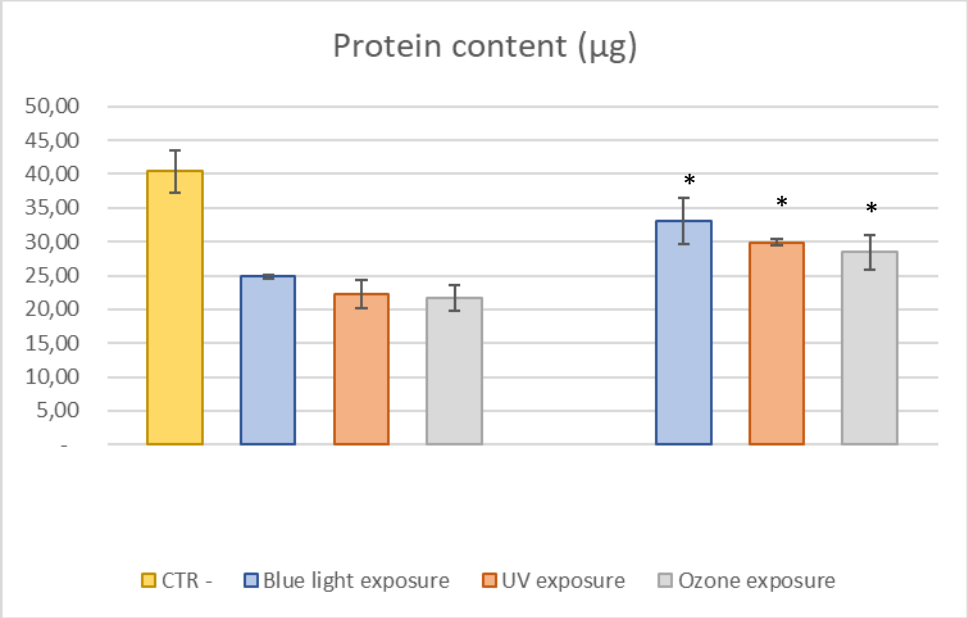
CELL METABOLISM

Table and graph 3

Protein content in CTR-, CTR+ and epidermis medium treated with the tested product. The results are expressed as mean ± st. dev. (µg proteine),mean % variation compared to CTR- and % protection compared to CTR+.

Protection vs Damaging agent - protein content (µg)

	Mean	St. Dev.	VAR% vs CTR-	% Protection	
CTR -	40,38	3,12			
Blue light exposure					
CTR+	24,83	0,27	-38,5%		
L'EAU DE JOUR N'02	33,08	3,38	-18,1%	20,44%	*
UV exposure					
CTR+	22,29	2,08	-44,8%		
L'EAU DE JOUR N'02	29,90	0,48	-25,9%	18,87%	*
Ozone exposure					
CTR+	21,65	1,92	-46,4%		
L'EAU DE JOUR N'02	28,48	2,52	-29,5%	16,90%	*



The tissue exposure to damage agents showed a significant decrease of the cell metabolism ($p<0.05$).
The tissues pretreated with the test item and then exposed to damage agents showed a statistically significant recovery of cell metabolism compared to the positive control (*$p<0.05$).

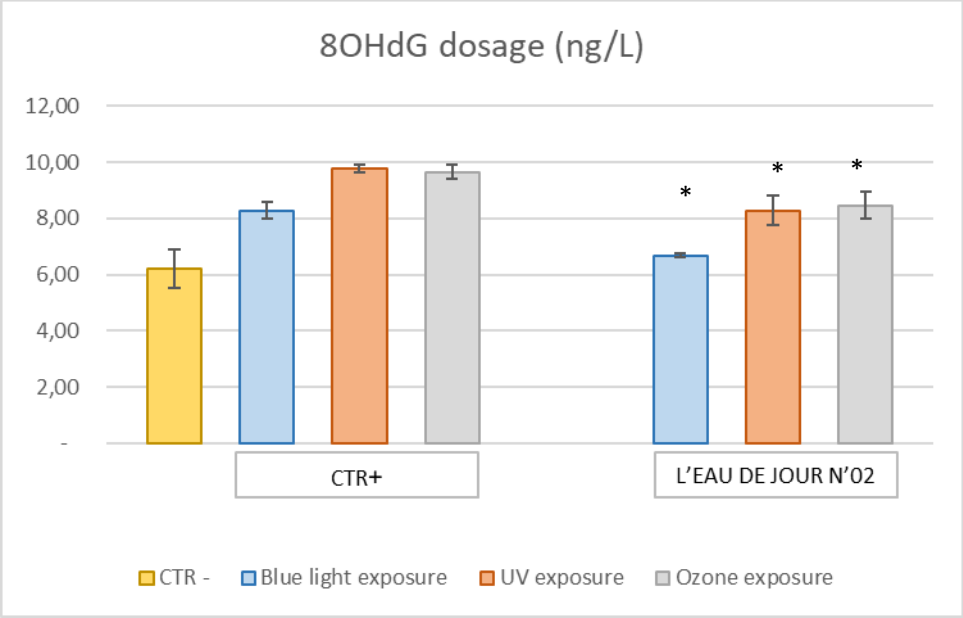
STUDY OF THE EXPRESSION OF 8OHdG

Table and graph 4

8OHdG levels in CTR-, CTR+ and epidermis treated with the tested product. The results are expressed as mean ± st. dev. (ng/L), mean % variation compared to CTR- and % protection compared to CTR+.

Protection vs Damaging agent - 8OHdG dosage (ng/L)

	Mean	St. Dev.	VAR% vs CTR-	% Protection	
CTR -	6,22	0,69			
Blue light exposure					
CTR+	8,28	0,29	33,0%		
L'EAU DE JOUR N'02	6,67	0,07	7,2%	25,83%	*
UV exposure					
CTR+	9,77	0,15	57,0%		
L'EAU DE JOUR N'02	8,28	0,52	33,0%	23,93%	*
Ozone exposure					
CTR+	9,65	0,25	55,1%		
L'EAU DE JOUR N'02	8,46	0,48	35,9%	19,18%	*



8OHdG levels, marker of DNA oxidative damage, increased after damage agents exposure (CTR +).
Tissue treated with the test item and then exposed to damage agents showed statistically significant lower levels of 8OHdG compared to the CTR+, indicating an efficacy of the tested product in DNA protection.
Moreover the level of 8-OHdG in the cells treated with tested product and exposed to blue light are similar to the level of 8-OHdG of CTR-.

3. CONCLUSIONS

According to the obtained data and referring to the applied experimental protocol, we can assess that

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highlighted a PROTECTIVE POTENTIAL by effectively keeping the cellular state, metabolism and oxidative stress and protecting the DNA of the reconstructed in vitro epidermis damaged by exposure to various damage agents

Sperimentatore | ***Experimenter***

Dott. Andrea POGGI

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Direttore dello Studio | ***Study Director***

Dott.ssa Silvana GIARDINA

Digitally signed by Silvana Giardina
Date: 2022.10.19 14:38:30 +02'00'

ANNEX I

Statement on report results extension to the formula lab-01414.24.

Based on the analysis of the qualitative and quantitative composition, the results reported in this study, performed on the formulation lab-01414.22, can be extended to the formulation lab-01414.24 (both containing the perfume Dreaming Night, in the formula lab-01414.22 at 0.2% and at in the formula lab-01414.24 at 0.1%).

INCI formula lab-01414.24*:* AQUA (WATER), PROPANEDIOL, NIACINAMIDE, 1,2-HEXANEDIOL, GLYCERIN, PROPYLENE GLYCOL, CAPRYLOYL/CAPROYL ANHYDRO METHYL GLUCAMIDE, FUMARIC ACID, PARFUM (FRAGRANCE), POLYDATIN GLUCOSIDE, CITRIC ACID, SODIUM CITRATE, METHYLGLUCOSIDE PHOSPHATE, COPPER LYSINATE/PROLINATE, CITRUS LIMON (LEMON) FRUIT EXTRACT, FUMARIA OFFICINALIS FLOWER/LEAF/STEM EXTRACT, DISODIUM ACETYL GLUCOSAMINE PHOSPHATE, CI 14700 (RED 4).

- Il risultato dello studio citato nel presente rapporto si riferisce esclusivamente al prodotto testato ed alle particolari condizioni sperimentali impiegate nello studio.
  The result of the study reported in this document is only referred to the tested sample and the specific experimental conditions.
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