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TEST ANTI-POLLUTION - Valutazione dell'azione protettiva *in vitro* di un prodotto cosmetico nei confronti dello stress ambientale mediante determinazione della vitalità cellulare e dosaggio dei radicali liberi

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ANTI-POLUTION TEST - In vitro evaluation of the protective action of a cosmetic product against environmental stress through determination of cell viability and dosage of ROS

PRIMA LUX SRL

SLAB002 - CREMA CORPO RASSODANTE ANTIPOLLUTION

Protocollo n° / *Report no.* **1802E31V2**

Luogo e data di emissione MILANO – 31 Luglio 2018
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RIASSUNTO

Il test per valutare l'azione protettiva del prodotto cosmetico nei confronti di agenti inquinanti e ambientali responsabili dell'invecchiamento cutaneo è stato condotto su cheratinociti umani trattati con 3 concentrazioni del prodotto da testare (1.0, 0.5 e 0.1 mg/ml) scelte dopo aver eseguito un test preliminare di citotossicità.
Le cellule sono state quindi stimolate con uno standard che riproduce l'inquinamento ambientale (urban dust), con uno standard di inquinamento indoor o esposte a raggi UVA allo scopo di "mimare" l'esposizione a cui quotidianamente è esposta la pelle. Cellule non trattate rappresentano il controllo negativo.
Si è quindi misurata la vitalità cellulare mediante test MTT e si sono dosati i radicali liberi (ROS) prodotti, confrontando i valori delle cellule non trattate con quelli delle cellule trattate con il prodotto testato.
Si è osservato nelle cellule irraggiate e trattate con il campione a tutte le concentrazioni testate la vitalità è superiore rispetto alle cellule irraggiate e non trattate. Nelle cellule irraggiate e trattate con campione a tutte le concentrazioni testate i livelli di ROS sono inferiori rispetto alle cellule irraggiate e non trattate.
Si può pertanto concludere che il prodotto testato ha un'attività "anti-pollution" che si manifesta in particolar modo nei confronti dell'irraggiamento UV.

ABSTRACT

The tests to evaluate the protective action of the cosmetic product against pollutants and environmental agents responsible for skin aging was conducted on human keratinocytes treated with 3 concentrations of the tested product (1.0, 0.5 and 0.1 mg/ml) chosen after a preliminary cytotoxicity test.
The cells were stimulated with a standard of atmospheric pollution (urban dust), with a standard of indoor pollution or exposed to UVA rays in order to "mimic" the exposure to which the skin is daily exposed. Untreated cells represent the negative control.
We measured the cell viability (through an MTT assay) and ROS production comparing the values of untreated cells with the one of cells treated with the tested product.
We observed that in the cells irradiated and treated with the sample at all tested concentrations the viability is higher compared to the irradiated and untreated cells. In the cells irradiated and treated with the sample at all tested concentrations, ROS levels are lower than in irradiated and untreated cells.
We can therefore conclude that the tested product has an "anti-pollution" activity, which is manifested in particular with regard to UV rays.

INTRODUCTION

Skin is the largest organ in body, and acts as the first and most important defense barrier against environmental contaminants. Skin is always exposed to the contaminants. Skin consists of three main layers: epidermis, dermis, and hypodermis. The stratum corneum of the epidermis is the outermost skin layer, acting as the main functional barrier.

Two main processes cause skin aging: extrinsic aging due to environmental factors and intrinsic aging due to time. Many reports suggest that environmental factors affect extrinsic skin aging, which is characterized by coarse wrinkles and unevenly distributed pigmentation. Intrinsic aging is related to the accumulation of cellular damage from the active oxygen radicals in the body. On the other hand, extrinsic aging is caused by harmful free radicals created by various environmental factors, including sun exposure and smoking. These radicals induce damage to the skin by causing an inflammatory reaction. Sun exposure (ultraviolet) is known as a major cause of aging: among all the environmental factors, UV radiation contributes up to 80%. It is the most important factor in skin aging, especially in premature aging. Both UVB (290–320 nm), and UVA (320–400 nm) are responsible, and the skin alterations caused by UV radiation depend upon the phenotype of photoexposed skin. UVB induces alterations mainly at the epidermal level, where the bulk of UVB is absorbed. It damages the DNA in keratinocytes and melanocytes. On the other hand UVA penetrates more deeply into the dermis and damages both the epidermis and dermis. The amount of UVA in ambient light exceeds the UVB by 10 to 100 times, but UVB has biological effects 1000 times stronger than UVA. It is accepted that UVA radiation plays an important role in the pathogenesis of photoaging.

However, it was reported that environmental contaminants, in particular particulate matter (PM) derived primarily from factories and motor vehicle exhaust, also significantly increased the symptoms of aging. The increased ROS decreases skin functions that prevent pathogen entry and repair DNA damage, resulting in the acceleration of the skin aging process. ROS production inhibits collagen synthesis as a result of activation of matrix metalloproteinases (MMPs). ROS increases MMP-12 to degrade collagen 5 and fibronectin. MMP-2, which is induced by ROS and UVA, is increased in fibroblasts, indicating degradation of the connective tissue matrix in the skin. Pro-inflammatory reactions by PM exposure are associated with skin aging as well. PM enhances pro-inflammatory cytokines, such as TNF-α, IL-1α, and IL-8, from human keratinocytes.

Another environmental factor contributing to premature aging is smoking. Tobacco smoking induces structural and compositional changes in the epidermis and dermis similar to those resulting from chronic UV radiation exposure, and it is an important environmental factor in premature skin aging. A smoker's face is characterized by gray skin (smoker's melanosis) and deep wrinkles (smoker's wrinkle). It seems that cigarette smoking induces the activation of MMPs in the same mode as in persons with significant sun exposure. Tobacco smoke extracts impair collagen biosynthesis significantly in cultured skin fibroblasts. In addition, production of the collagen precursors, procollagen types I and III, was decreased significantly in supernatants of cultured fibroblasts treated with tobacco smoke extracts.

SCOPO

Lo scopo del test è quello di valutare la capacità protettiva del prodotto testato nei confronti di contaminanti atmosferici outdoor e indoor e delle radiazioni UV

AIM

The aim of the test was to evaluate the protective capacity of the tested product against atmospheric outdoor e indoor pollutants and UV radiation.

Conditions of stimulation

In order to simulate a condition of environmental stress, the cells were stimulated with a standard of urban pollution (urban dust), cigarette smoke and subjected to irradiation with UVA rays.

1. ***Urban dust.*** *The urban dust is a standard reference material containing polyciclic aromatic hydrocarbons (PAHs), nitro-substituted PAHs, polychlorinated biphenyl (PCB), chlorinated pesticides and inorganic heavy metals. The standard was prepared from atmosferic particulate material collected in the Washington DC area over a period longer than 12 months using a baghouse specially designed for the purpose. The urban dust standard is not intended to be representative of the area in which it was collected, but it should generally tipify atmospheric particulate matter obtained from an urban area.*
2. ***Indoor standard.*** *The indoor standard is a mixture of 52 components achieved analizying the vacuum cleaner bags obtained from households, cleaning services, motels, and hotels from North Carolina, Maryland, Ohio, and New Jersey.*
3. ***UV rays.*** *The lamp used in the experiments is a solar light simulator. A constant emission in the UVA range (315-400 nm) with an irradiance of 1.7 mW/cm^2 is assured. The UVB emission is screened in order to avoid direct cell mortality.*

Test execution

We performed a preliminary MTT test in order to choose non-cytotoxic concentrations of tested product to be used: the cells were treated with several dilutions of the tested sample. Untreated cells maintained in growth medium represent the negative control. After 24 hours of contact, the cells were washed with phosphate buffered saline (PBS) and subjected to MTT assay.

For the "anti-pollution" test, an adequate number of cells was seeded in 96-well plates. Once reached a semi-confluent monolayer, the cells were treated with the chosen concentrations of the tested sample and different stimuli able to reproduce the skin exposure to atmospheric pollutants. Intensity stimuli were chosen in order to cause significant cell damage (reduction of viability by at least 20-30%). Untreated cells kept in culture medium represent negative controls. At the end of an overnight contact period the cells were washed with a phosphate buffer (phosphate buffer saline, PBS) in order to eliminate any treatment residues and then subjected to MTT tests to measure residual viability and to the dosage of ROS with DCFA.

CONCLUSIONI

Il campione denominato

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ha azione protettiva nei confronti dello stress ambientale su cheratinociti *in vitro*.
Tale azione di manifesta in particolare nei confronti dell'irraggiamento UV

CONCLUSIONS

The sample called

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has protective action against environmental stress on keratinocytes in vitro.
This action is particularly evident with regard to UV rays

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CDC Istituto di Ricerche Dermo-Cliniche

Valutazione dell'effetto e della gradevolezza di un prodotto cosmetico mediante test clinico

Evaluation of the effect and of the acceptability of a cosmetic product through a clinical test

PRIVILEGE ONE CONCENTRATE GREAT THE BODY ESSENTIAL
lotto 260618

PRIMA LUX SRL

Protocollo n° / *Report no.* **1802E29F**

Luogo e data di emissione: MILANO – 24 Agosto 2018
Place and date of issue: MILAN – 24th August 2018

SOMMARIO

Il prodotto cosmetico oggetto del presente test è stato sottoposto ad indagine clinica per valutare se possieda effetto elasticizzante e rassodante e per valutarne la gradevolezza all'uso.
Il test è stato condotto da un dermatologo membro dello staff Bio Basic Europe ed è così articolato: sono state selezionate 20 volontarie aventi età compresa tra i 16 ed i 60 pelle poco tonica, cui è stato chiesto di applicare il prodotto in oggetto sul corpo, una volta al giorno per 28 giorni consecutivi. Durante questo periodo è stata valutata strumentalmente l'elasticità cutanea e clinicamente il rassodamento cutaneo.
Alla fine del test sono state poi raccolte una serie di valutazioni sensoriali espresse dalle stesse volontarie. Per l'autovalutazione si è utilizzata la scala VNS con valori da 0 a 10.
In base ai risultati ottenuti possiamo affermare che il prodotto cosmetico testato ha dimostrato possedere un effetto elasticizzante e rassodante cutaneo, nelle volontarie sottoposte a test clinico.
Il prodotto ha inoltre dimostrato possedere una buona gradevolezza all'uso.

SUMMARY

The purpose of this clinical test is to evaluate if the tested cosmetic product has an elasticizing and firming effect and to evaluate its acceptability to use.
This test was performed by a dermatologist member of Bio Basic Europe staff: 20 female panellists, with an age between 18 and 60 years and with low toned skin were recruited and were asked to apply the cosmetic product on the body, once per day for 28 consecutive days. During this period, skin elasticity has been instrumentally evaluated and skin firmness has been clinically evaluated.
All the evaluations given by volunteers in the sensorial test were collected at the end of this test.
The score they gave is according to VNS scale (0-10 where 0 is the minimum value and 10 is the maximum one).
According to the obtained results we can state that, in the volunteers who underwent the clinical test, the tested product has proved to have a skin elasticizing and firming effect.
The product has also proved to have a good acceptability.

PARTE SPERIMENTALE

EXPERIMENTAL PART

Protocollo n° 1802E29F

Report no. 1802E29F

Titolo

Valutazione dell'effetto e della gradevolezza di un prodotto cosmetico mediante test clinico

Title

Evaluation of the effect and of the acceptability of a cosmetic product through a clinical test

Scopo

Il test consente di valutare se il prodotto cosmetico sottoposto a tale test possieda effetto elasticizzante e rassodante cuteneo. Il test fornisce inoltre informazioni sulla gradevolezza d'uso del prodotto.

Aim

The purpose of this test is to evaluate whether the tested cosmetic product has a skin elasticizing and firming effect. Product acceptability is evaluated too.

Informazioni legali

In accordo alla normativa vigente, e alla dichiarazione di Helsinki, i volontari sono adeguatamente informati circa lo scopo, le modalità e le caratteristiche dello studio clinico, gli effetti favorevoli ed i possibili effetti collaterali. Ciascun volontario firma per accettazione un modulo di consenso informato, gestito ed archiviato in accordo alle procedure interne del Sistema Gestione Qualità di Bio Basic Europe S.r.l.

Legal information

In accordance with the current legislation and the declaration of Helsinki, all volunteers must be adequately informed of the aims, methods, clinical trial details, anticipated benefits and potential undesirable effects of the study. Each panellist must sign an informed consent form, which is managed and archived by applying the internal procedure of the Quality Management System of Bio Basic Europe S.r.l.

Informazioni contrattuali

- Relazione tecnica eseguita come da contratto tra BIO BASIC EUROPE S.r.l. ed Università degli Studi di Pavia.
- Stesura del report eseguita presso BIO BASIC EUROPE S.r.l. per conto di PRIMA LUX SRL
- Sperimentazione eseguita presso CDC - Istituto di Ricerche Dermo-Cliniche

Contract information

- *Technical report performed by BIO BASIC EUROPE S.r.l. and Università degli Studi di Pavia.*
- *Final technical report written by BIO BASIC EUROPE S.r.l. on behalf of PRIMA LUX SRL*
- *Experimentation performed at CDC - Dermo-clinic Research Institute*

CARATTERISTICHE DELLO STUDIO

Soggetti del test

Sono stati selezionati 20 soggetti di sesso femminile, aventi età compresa tra i 18 ed i 60 anni, secondo i seguenti criteri di inclusione:

- pelle poco tonica

- Buono stato di salute generale/assenza di disturbi psicologici e/o cognitivi;
- Assenza di patologie dermatologiche ed allergologiche (cosmetologiche o ad altri eccipienti specifici), o altre patologie (tipo reazione irritative di origine non nota);
- Assenza di trattamenti farmacologici in atto che possano influire sull'esito del test;
- Non partecipazione ad altri studi clinici nei 30 giorni precedenti;
- Ottenimento del consenso informato.

Preparazione dei campioni

I campioni sono stati applicati, in funzione delle loro caratteristiche d'uso: tal quale.

Metodo di applicazione dei campioni

I campioni sono stati applicati sul corpo, una volta al giorno per 28 giorni consecutivi.

CLINICAL TEST FEATURES

Test subjects

20 female subjects, with an age between 18 and 60 years, have been selected for the test, following the undermentioned inclusion criteria:

- *low toned skin*

- good state of health/absence of psychological and/or cognitive disorders;
- no dermatopathies and allergic pathologies (to cosmetics or other specific excipient), or other pathologies (as unknown irritant responses);
- no ongoing pharmacological treatments that could affect the result of the test;
- no participations in other clinical trial during the previous 30 days;
- signature of the informed consent form.

Preparation of the samples

Samples of the products have been applied following their usual use: as they are.

Method of application of the samples

Samples of the tested product have been applied on the body, once per day for 28 consecutive days.

EXECUTION OF THE TEST

During the test, the following parameters have been evaluated:

INSTRUMENTAL PARAMETERS

*- The **elasticity** was measured with the elastometer CUTOMETER® – MPA 580*

Skin elasticity was measured with Cutometer®. Before the test, it was necessary to standardize the measuring technique, of the level (maximum 500 mBar) and of the time of suction, with subsequent release, as well as the number of repeated measurements in the same test area (maximum 9). For this research, a suction cycle of 1 second at 500 mBar followed by a releasing cycle of one second was selected.
Regarding the elastometric measurement, the skin surface was aspirated from the depression induced by the machine into the aperture of the elastometer's measuring probe.
Depth of skin penetration inside the probe was measured by an optic sensor.
Linkage to a PC allowed the data obtained to be displayed, stored and printed. Cutaneous elasticity reflects the skin's potential capacity (measured in mm) for retraction.
A graph shows the deformation curve of skin undergoing aspiration, and includes two components (see the figure).

- *An elastic component (Ue), which corresponds to the part of the curve that rises rapidly, and is reversible to the deformation;*
- *A plastic component (Uv), which corresponds to the part of the curve that rises slowly and is not completely reversible to the deformation.*

❖ *During the releasing phase, the quantity of deformation remaining in the skin can be observed (Ua-Ur=residual deformation).*
Cutaneous elasticity is defined as the ratio:

Elasticity= Ua/Uf (R2)

Which represents the recovery degree of the maximum deformation reached, whose values range between 0 and 1 (maximum elasticity).

CLINICAL PARAMETERS

Evaluations performed:
- skin firmness

The readings and the evaluations have been performed on the forearm:
- *at [t0] (basal value)*
- *after product use: after 14 days [t14] and after 28 days [t28]*

SELF-EVALUATIONS

Volunteers opinions are also taken after product use: after 28 days [t28].
This self-evaluation was performed according to VNS scale where 0 is the minimum value and 10 is the maximum value.

CONCLUSIONI

In base ai risultati ottenuti possiamo affermare che il prodotto cosmetico:

CONCLUSIONS

According to the obtained results we can state that the cosmetic product:

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ha dimostrato possedere un effetto elasticizzante e rassodante cutaneo, nelle volontarie sottoposte a test clinico. Il prodotto ha inoltre dimostrato possedere una buona gradevolezza all'uso.

in the volunteers who underwent the clinical test, the tested product has proved to have a skin elasticizing an firming effect.
The product has also proved to have a good acceptability.

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