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"Ozone peaks as a major emerging stressor and pollutant causing detrimental skin conditions; A skincare serum offers protection and correction."

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Abstract

Major air pollutants which affect the skin are UV rays, visible light, particulate matter (PM), heavy metals and, more recently highlighted but among the most toxic, ozone (O_3). Tropospheric ozone is an atmospheric pollutant created by chemical reactions between NOx gases (oxides of nitrogen produced by combustion) and volatile organic compounds. The combination of these chemicals in the presence of sunlight form ozone. Tropospheric ozone has deleterious effects on human health and is a key factor in skin damage.

In the present work, we evaluated whether photo-pollution including O3 acts in inducing skin damage, and whether this effect could be prevented through native *Crithmum maritimum* cells extract or a topical application of a specific skincare SERUM containing it.

- A preliminary *in-vitro* study carried out on complete reconstructed skin models in the presence of O3, revealed a reduction in the oxidant marker 8-isoprostane thanks to the systemic application of native *Crithmum maritimum* cells, demonstrating their protective antioxidant efficacy.

- An *ex-vivo* test conducted on skin explants highlighted the additive effect of O3 to photo-pollution in increasing levels of oxi-inflammatory markers and demonstrated a biological antioxidant and anti-inflammatory preventive efficacy of the skincare SERUM apply topically.

- A clinical study conducted in harsh urban pollution including ozone pics in Shijiazhuang, China, proved after 6 months that the SERUM decreased significantly skin imperfections and aging signs such as wrinkles and improved skin elasticity and radiance.

Keywords: Anti-aging, External stress, Pollution, Skin texture

1. Introduction

The skin, being the largest and outermost organ, is the main barrier of our body against the external environment and is therefore the main target for the outdoor stressors [1, 2].

Major air pollutants which affect the skin are UV rays, visible light, particulate matter (PM), heavy metals and, more recently highlighted but among the most toxic, ozone (O3). Ozone is an atmospheric pollutant well known for its deleterious effects on human health. It forms in the lower layers of the atmosphere from the chemical transformation of nitrogen oxides and volatile organic compounds, mainly emitted by road traffic, industrial activities, domestic activities but also vegetation cover under the effect of solar radiation and high temperatures.

This pollutant tropospheric O3, not to be confused with the protective stratospheric O3, is the only pollutant increasing in most industrialized countries in recent decades and is a key factor in skin damage [3-4].

By inducing oxidative stress and inflammation in the skin [5], O3 is a key factor in skin damage.

In the present work, we evaluated thanks to 3 complementary studies (*in-vitro, ex-vivo* and *in-vivo*) whether photo-pollution including O3 acts in inducing skin damage, and whether this effect could be prevented through native *Crithmum maritimum* cells extract or a topical application of a specific skincare SERUM containing it.

2. Materials and Methods

2.1. *In-vitro* study

A preliminary *in-vitro* study was carried out on reconstructed human full-thickness skin models T-Skin™ (Episkin SA, Lyon, France) to demonstrate the protective efficacy of native *Crithmum maritimum* cells by systemic application against O3 on the skin, thanks to the evaluation of the 8-isoprostane marker.

In-vitro study was conducted on reconstructed human full-thickness skin models either untreated and non-exposed to stress or exposed to stress (O3) or treated with systemic application of native *Crithmum maritimum* cells and non-exposed to stress or exposed to stress (O3).

		D18	D19	D20	D21	D22
T	Tissu control					▼
CM	Native *Crithmum maritimum* cells systemic application	▲	▲	▲		▼
S	STRESS: OZONE	■		■		▼
CMS	Native *Crithmum maritimum* cells + STRESS	▲ ■	▲	▲ ■		▼

▲ Native *Crithmum maritimum* cells systemic application ■ STRESS exposure: OZONE ▼ Explant sampling

Table I: Reconstructed human full-thickness skin model distribution & schedule of *in-vitro* study

Culture of reconstructed full-thickness skin model

Reconstructed human full-thickness skin models were shipped after 11 days of culture (D11), receipt at D12 and cultured according to the manufacturer's instructions.

Active ingredient application

Batches CM and CMS: native *Crithmum maritimum* cells dosed at 0.04% were systemically applied, in 2.5 ml maintenance medium.

Reconstructed human full-thickness skin model exposure to ozone (O3)

Ozone exposure of reconstructed skin model was performed thanks to a system combining a cell culture incubator and an ozone generator. This system allowed a controlled ozone generation, a homogeneous gas dispersion and a reduction of ozone interaction with materials other than reconstructed skin model, while maintaining optimal skin culture conditions. In this system, T-skin™ model culture conditions were optimized. Ozone was generated with the 49i UV photometric O3 Analyzer (Thermo Scientific, Mégatec), using a UV lamp photolyzing oxygen from air. T-skin™ were exposed to 0.4 ppm of O3 for 18 hours, with 2 repeated exposures. Subsequently, the medium was daily replaced with fresh maintenance medium. Temperature, CO2 percentage and humidity were monitored during exposures (37°C, 5% and 95%, respectively). T-Skin™ were exposed to O3 between D18 and D21 and samples were collected at D22 (Table I, batches S and CMS).

Quantification of 8-isoprostane marker

The evaluation was done by immunostaining and image analysis.

8-isoprostane was measured in culture medium collected at D22, with the 8-isoprostane ELISA kit (Cayman Chemical). The ELISA was performed according to the manufacturer's instructions. The 8-isoprostane standards included in the ELISA kit were treated in the same way as the samples to obtain a calibration curve, which was used to estimate the 8-isoprostane levels in the samples.

Statistical analysis

One-way analysis of variance (ANOVA Dunnett corrected) was used. The results were considered significant with a P-value<0.05. Data are expressed as mean ± S.D. of triplicate determinations obtained in 2 independent experiments.

2.2. *Ex-vivo* study

An *ex-vivo* study mimicking extreme environmental conditions (pollutants + UVA + blue light + O3 exposure) was set up on skin explants to highlight the additive effect of O3 to photo-pollution in increasing levels of oxi-inflammatory markers and the SERUM biological preventive efficacy evaluating the 4HNE and IL1-alpha markers.

Ex-vivo study was conducted on skin explants either untreated and non-exposed to stress, exposed to STRESS 1 (Pollubox® + UVA + blue light + O3) or exposed to STRESS 2 (Pollubox® + UVA + blue light) or treated with the skincare SERUM and non-exposed to stress or exposed to STRESS 1 (Pollubox® + UVA + blue light + O3). Each of these batches was compared to “young” skin explants untreated and non-exposed to stress.

18 human skin explants of an average diameter of 11 mm (±1mm) were prepared on an abdominoplasty coming from a 51-year-old Caucasian woman with a phototype II* and 6 human skin explants of an average diameter of 11 mm (±1mm) were prepared on an abdominoplasty coming from a 18-year-old Caucasian woman with a phototype III*.

*According to Fitzpatrick skin type classification

		D0	D1	D2	D3	D4
T01	Tissu control “old” donor	▼				
T1	Untreated control “old” donor					▼
P	SERUM	▲	▲	▲	▲	▼
S1	STRESS 1 exposure: POLLUBOX® + UVA + BLUE LIGHT + OZONE				■	▼
PS1	SERUM + STRESS 1 exposure	▲	▲	▲	▲ ■	▼
S2	STRESS 2 exposure: POLLUBOX® + UVA + BLUE LIGHT				◉	▼
T02	Tissu control “young” donor	▼				
T2	Untreated control “young” donor					▼

▲ Skincare SERUM application ■ STRESS 1 exposure: POLLUBOX® + UVA irradiation + BLUE LIGHT irradiation + OZONE ◉ STRESS 2 exposure: POLLUBOX® + UVA irradiation + BLUE LIGHT irradiation ▼ Explant sampling

Table II: Explants distribution & schedule of *ex-vivo* study

Products application

Batches P1 and P1S1: SERUM was topically applied in the morning, on the basis of 2 µl per explant of 1 cm^2 (≈ 2 mg/cm^2).

Stress exposure

Batches S1, P1S1 & S2 were put in HBSS (Hanks' balanced salt solution) medium and exposed to successive environmental stresses: Pollubox® (pollutant mixture), followed by UVA & blue light irradiations and OZONE exposure (STRESS 1) or Pollubox® (pollutant mixture), followed by UVA & blue light irradiations (STRESS 2).

Pollubox® is an exposure chamber where only the surface of human skin explants is exposed to environmental pollutants such as PM 2.5, hydrocarbons (benzene, xylene & toluene) and heavy metals, to represent the polluted environment of important cities in an *ex-vivo* setting. Pollutants were vaporised into cloud form on human skin explants during 1.5 hours.

UVA irradiation was performed with a UV simulator Vilber Lourmat® (Marne-la-Vallée, France) RMX 3W with a dose of 9 J/cm2 of UVA during 30 minutes.

Blue light irradiation was carried out with the Solarbox® device for 2h at a dose of 42.5 J/cm².

Ozone exposure was generated by an Ozonator at a dose of 3 to 3.5 ppm during 30 minutes.

The evaluation was done by immunostaining and image analysis.

The immunostaining was performed with a polyclonal anti-4-HNE antibody (Abcam, ab46545, Paris, France) and a monoclonal anti-IL-1α antibody (Novus Biologicals, NBP2-45400, clone OTI2F8, Minneapolis, USA).

The immunostaining was assessed by microscopical observation and semi-quantified by image analysis using the software cellSens (Olympus, Rungis, France).

For each batch of explants, the percentage of the region of interest (ROI) covered by the staining was determined by image analysis. The stained surface percentage for the treatment was compared to the untreated condition T or to the stress-induced condition S.

Statistical analysis

Statistical analysis was performed using classical Student t-test. The tests were carried out in bilateral mode on two samples of supposedly different variances (heteroscedastic).

Student t-test gives the probability "*p*" for two batches to be significantly different. The difference between two batches is significant if $p<0.01$(**), so a probability of 99% for two batches to be significantly different or $p<0.05$(*), so a probability of 95% for two batches to be significantly different.

2.3. *In-vivo* study = clinical study

A 6-months clinical study was conducted on 2 groups (Group 1: SERUM vs Group 2: neutral cream) of 42 & 43 Asian volunteers, from 25 to 45 years old, in harsh urban pollution including ozone pics during hot conditions in Shijiazhuang, China to prove the SERUM effectiveness regarding skin imperfections and aging signs.

Inclusion criteria were as follows:

- ≥ 3 inflammatory lesions (for at least 30% of volunteers)
- Sensitive skin (according to sensitive questionnaire L'OREAL [6])

- Oily or combination to oily skin
- Underneath eye wrinkles (score ≥ 1.5 from Atlas Vol. 2 - Asian type [7] - on at least one side of the face)
- Uneven skin tone (score ≥ 3 from a 10-point scale of skin tone evenness)
- Living in Shijiazhuang for at least 15 years.

After a 15 days wash-out, skincare SERUM (Group 1) or neutral cream (Group 2) were applied twice a day during 6 months.

Assessments were carried out after 1, 3 and 6 months of product application by counting of lesions (inflammatory & retentional lesions), instrumental measurements (Sebumeter®, Chromameter® and Cutometer®), clinical scorings (Atlas & score scales), self-assessments (score scales) and satisfaction questionnaires.

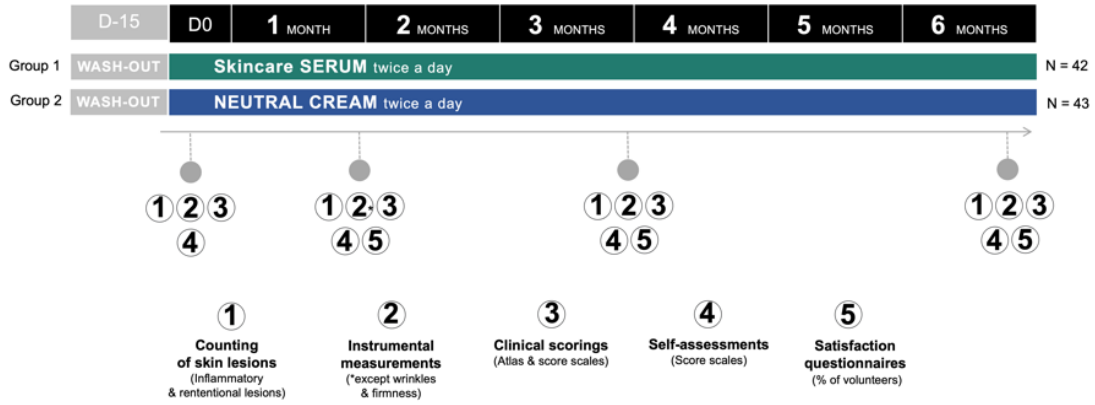

Figure 1: Clinical study design

Statistical analysis

The statistical methods of each measurement item were shown in Table III.

Measurement item	Comparison of time effect (T0 vs Tn)	Treatment effect (Group 1 vs Group 2)
Counting of skin lesions	Dunnett-t test (Normal distribution) / Wilcoxon test (Abnormal distribution)	t-test (Normal distribution) / Mann-Whitney U test (Abnormal distribution)
Clinical scorings	Wilcoxon test	Mann-Whitney U test
Self-assessments	Wilcoxon test	Wilcoxon test
Sebumeter®	Wilcoxon test	t-test (Normal distribution) / Mann-Whitney U test (Abnormal distribution)
Chromameter®	Dunnett-t test	t-test (Normal distribution) / Mann-Whitney U test (Abnormal distribution)
Cutometer®	t-test (Normal distribution) / Wilcoxon test (Abnormal distribution)	t-test

Table III: Summary of statistical methods for each measurement item.

3. Results

3.1. *In-vitro* study

Looking to *in-vitro* study, combining 3 days of systemic application of native *Crithmum maritimum* cells and O3 stress exposure, native *Crithmum maritimum* cells induce a significant decrease of oxidative markers 8-isoprostane of -18% (CMS *vs S*).

3.2. *Ex-vivo* study

Comparing exposure to STRESS 1 containing ozone *versus* STRESS 2 without ozone, ozone multiplies by 1.6 the oxidative marker 4-HNE and by 2.5 the inflammatory marker IL1-alpha (S1 *vs* S2).

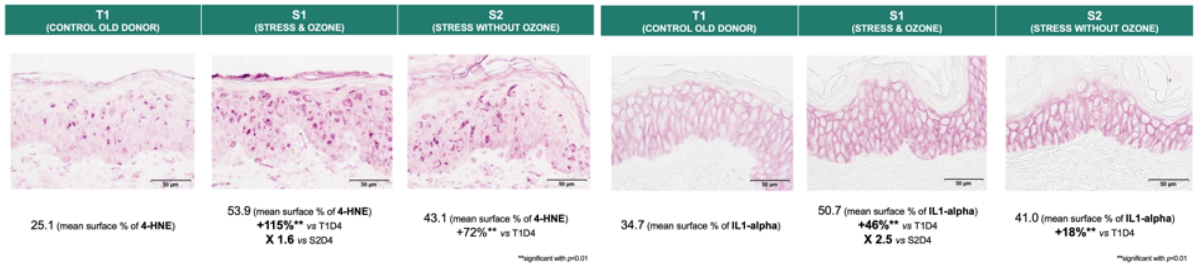

Figure 2: *Ex-vivo* study results - impact of O3 on 4-HNE & IL1-alpha

Comparing 4 days of treatment with the skincare SERUM following by STRESS 1 exposure including ozone to STRESS 1 exposure alone, skincare SERUM application induces a decrease of the oxidative marker 4-HNE of -37% and reduces the inflammatory marker IL1-alpha by -49% (PS1 *vs* S1). The concentration of the inflammatory marker was even lower than the unstressed “older” skin control (PS1 vs T1) and back to the level of “young” skin (no significant difference between PS1 & T2).

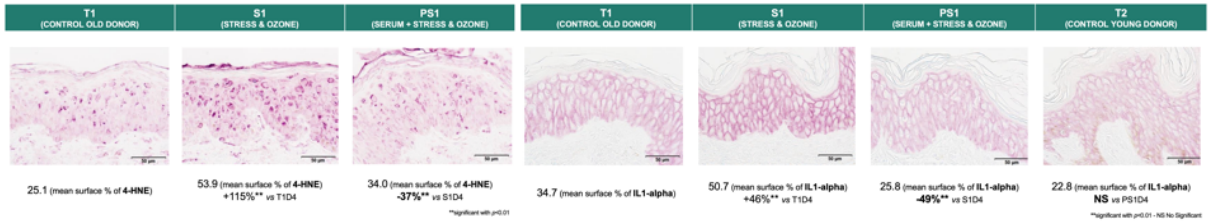

Figure 3: *Ex-vivo* study results - impact of the skincare SERUM application on 4-HNE & IL1-alpha after stress exposure (including O3)

3.3. *In-vivo* study = clinical study

In-vivo, regarding skin imperfections, compared to the neutral cream, total number of lesions (retentional & inflammatory) is reduced by -32.87% after 1 month, -58.47% after 3 months and -71.61% after 6 months. Sebum level measured with the Sebumeter® decreases by -16.34% from 1 month and -16.40% after 3 months. Clinical scoring of skin greasiness / shininess decreases by -21.87% after 3 months.

Variation percentage	D0	1 MONTH	3 MONTHS	6 MONTHS	1 MONTH vs D0	3 MONTHS vs D0	6 MONTHS vs D0
IMPERFECTIONS **Total number of lesions** - **Inflammatory & retentional lesions** (counting)							
GROUP 1	23.90	16.05	9.93	6.79	**-32.87 %*** *	**-58.47 %*** *	**-71.61 %*** *
GROUP 2	25.44	22.53	20.56	14.95	-11.43 %*	-19.20 %*	-41.22 %*
Sebum level on forehead (Sebumeter®)							
GROUP 1	108.14	90.48	90.40	95.74	**-16.34 %*** *	**-16.40 %*** *	**-11.47 %*** *
GROUP 2	108.63	103.88	110.74	108.26	NS	NS	NS
Skin greasiness / shininess (clinical scoring)							
GROUP 1	4.85	4.18	3.79	3.52	**-13.76 %***	**-21.87 %*** *	**-27.27 %***
GROUP 2	4.81	4.35	4.26	1.18	9.66 %*	-11.59 %*	-21.26 %*

*Significance p<0.05

SIGNIFICANT DIFFERENCE BETWEEN THE 2 PRODUCTS IN FAVOUR OF **POWERCELL SKINMUNITY SERUM** *

Table IV: *In-vivo* study results on skin imperfections after 1, 3 & 6 months of skincare SERUM vs neutral cream application.

Figure 4: *In-vivo* study results on skin greasiness / shininess - best case

Concerning skin aging signs, always compared to the neutral cream, wrinkles on forehead are reduced by -19.59% after 6 months (clinical scoring - Atlas). Skin elasticity measured with the Cutometer® increases by +10.52% after 6 months, confirmed by clinical scoring increase of +12.01% after 3 months and +24.80% after 6 months. Skin homogeneity measured with the Chromameter® increases by +19.54% after 6 months and skin radiance increases by +20.47% after 6 months (clinical scoring).

Variation percentage	D0	1 MONTH	3 MONTHS	6 MONTHS	1 MONTH vs D0	3 MONTHS vs D0	6 MONTHS vs D0
AGING SIGNS **Wrinkles** on forehead (clinical scoring - Atlas)							
GROUP 1	1.87	1.69	1.60	1.50	**-9.67 %***	**-14.25 %***	**-19.59 %*** *
GROUP 2	2.19	2.11	2.05	2.02	-3.82 %*	-6.37 %*	-7.86 %*
Skin elasticity (Cutometer®)							
GROUP 1	48.73	-	48.70	53.85	-	NS	**+10.52 %*** *
GROUP 2	49.44	-	49.13	50.07	-	NS	NS
Skin elasticity (clinical scoring)							
GROUP 1	4.56	4.32	4.01	3.43	**+5.22 %***	**+12.01 %*** *	**+24.80 %*** *
GROUP 2	4.48	4.38	4.22	3.99	+2.08 %*	+5.71 %*	+10.91 %*
Skin homogeneity on cheek (Chromameter®)							
GROUP 1	3.00	2.84	2.43	2.41	NS	**+18.92 %***	**+19.54 %*** *
GROUP 2	3.17	3.06	2.78	2.86	NS	+12.29 %*	+9.71 %*
Skin radiance (clinical scoring)							
GROUP 1	4.54	4.38	4.05	3.61	**+3.41 %***	**+10.76 %***	**+20.47 %*** *
GROUP 2	4.72	4.69	4.48	4.17	NS	+5.17 %*	+11.58 %*

*Significance p<0.05

SIGNIFICANT DIFFERENCE BETWEEN THE 2 PRODUCTS IN FAVOUR OF **POWERCELL SKINMUNITY SERUM** *

Table V: *In-vivo* study results on skin aging signs after 1, 3 & 6 months of skincare SERUM vs neutral cream application.

Figure 5: *In-vivo* study results on forehead wrinkles - best case

All these results were observed and confirmed by the volunteers.

4. Discussion

This combination of *in-vitro, ex-vivo* and *in-vivo* studies confirmed the harmful impact of tropospheric ozone on the skin and highlighted the effectiveness of native *Crithmum maritimum* cells and a serum containing them, both in terms of the underlying biological mechanisms and the visible signs on the skin.

The *in-vitro* test, carried out in the presence of O3, revealed a reduction in the oxidant marker 8-isoprostane thanks to the systemic application of native *Crithmum maritimum* cells, demonstrating their protective antioxidant efficacy.

The *ex-vivo* test highlighted the additive effect of O3 to photo-pollution in increasing levels of oxi-inflammatory markers and demonstrated a biological antioxidant and anti-inflammatory preventive efficacy of the skincare SERUM apply topically.

The clinical study conducted on Asian women in harsh urban pollution including ozone pics in Shijiazhuang, China, proved after 6 months of application that the SERUM decreased significantly skin imperfections and aging signs such as wrinkles, and improved skin elasticity and radiance.

5. Conclusion

Faced with increasing environmental photo-pollution, it is essential to develop cosmetic skincare designed to counter oxidative stress and the inflammatory phenomena induced, to limit skin imperfections and reduce aging signs.

These studies investigated the combined effects on human skin of the most harmful outdoor stressors, including O3 among the most toxic, and the only pollutant increasing in most industrialized countries in recent decades. There suggested that native *Crithmum maritimum* cell prevents deleterious effect of O3 and that topical application of a skincare SERUM containing native *Crithmum maritimum* cells prevents inflammation and oxidation leading to decrease skin imperfections and aging signs.

Conflict of Interest Statement

The research was funded by HELENA RUBINSTEIN. The authors report no other conflicts of interest in this work.

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