How to take care of the window of our soul?
Gentiopicroside-rich *Gentiana lutea* extract: an integrative solution for the eye contour

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Abstract

Background: Systemic eye rejuvenation aims to improve the appearance of periorbital problems: hyperpigmentation, eye bags, eyelid sagging and wrinkles. In this study, *Gentiana lutea* Extract (GlE) containing gentiopicroside (65% per dry matter) was evaluated in *in vitro, ex-vivo* and *in vivo* studies for its activity to create a multifunctional effect on eye contours.

Methods: The effect of GlE on antioxidant, inflammation and angiogenesis responses was evaluated *in vitro* by RT-qPCR after treatment of NHDF and by evaluating VEGF-induced pseudotube formation in a co-culture system. The effect of GIE on VEGF-C protein release and collagen I production by dermal fibroblasts was also done. Expressions of carboxymethyl-lysine (CML) and Glyoxalase-1 (Glo-1) were quantified on skin explants topically treated with GlE in basal and UVA-irradiated conditions. An *in vivo* study was conducted using topical twice daily for 14 days on eye area (split-face application: cream containing 0.5% of GIE versus placebo). 3D image acquisition and skin color measurement were performed at D0 and D14.

Results: GIE targeted AGEs pathways which stimulate inflammation pathways. It upregulated Nrf2 antioxidant pathway and decreased angiogenesis through the reduction of formation of pseudotubes. GIE increased significantly both VEGF-C release (48%: $p<0.01$) and collagen I (76%: $p<0.05$) compared to untreated fibroblasts. Significant reduction of CML expression ($p<0.001$) following UVA irradiation were noted compared to control. In contrast, Glo-1 was significantly increased (35%: $p<0.01$).

Conclusion: Eye cream containing a natural extract achieved skin rejuvenation by improving appearance of wrinkled eyelids, hyperpigmentation and puffiness after 14 days of application.

Keywords: anti-aging; eyes; gentiopicroside; Advanced Glycation End-products.

Introduction

The special senses – sight, hearing, smell, touch, and balance – allow us to perceive the world and communicate. Like all body systems, they undergo age-related changes that negatively affect their function [1]. Focusing on the eyes, they are considered to be the window of the soul and the focal point of social interaction [2]. Moreover, if the eyes are healthy, the whole body will be full of light. Take care of this area is thus very important, even more so after the pandemic we are going through, screen time due to work from home and binge watching having led to dark circles and wrinkles. Because of this, the under-eye area looks worn out and old.

Signs of ageing notoriously appear earlier around the periorbital area than in other parts of the face due to a unique combination of thin skin (the eye contour has the thinnest skin of the facial area), perpetual movements (over 10 000 blinks per day in addition to 22 muscles in constant motion), and decreased amount of subcutaneous fat.

Even if the physiopathology of skin ageing is multifactorial, cell degeneration involving the accumulation of advanced glycosylation end products (i.e., Advanced Glycation End-products -AGE) is a key factor [3]. Indeed, production of AGEs on collagen leads to cross-linking inducing abnormalities in the extracellular matrix, impairing cell-matrix interaction and leading to skin sagging. AGEs also bind to specific receptors on immune cells, which triggers the release of inflammatory mediators and generation of Reactive Oxygen Species (ROS) leading to increase in the production of AGEs damage. AGEs also modulate the function of endothelial cells affecting the expression of angiogenic factors by increasing Vascular Endothelial Growth Factor-A (VEGF-A), inducing an increase in capillary permeability [4].

Periorbital edema, as well as erythema can also be linked to rubbing and scratching the skin around the eyes (mechanical friction) or to conditions such as post-inflammatory hemodynamic congestion, and impaired lymphatic circulation. The lymphatic system plays an important role

in tissue fluid homeostasis, and immune surveillance. Lymphatic vessels indeed are heavily involved in regulating the inflammatory response by draining extravasated fluid, antigens, inflammatory mediators, and antigen-presenting cells from the periphery to the lymph nodes, where specific immune responses are mounted [5]. In inflamed tissue, the lymphatic vasculature undergoes extensive remodeling and expansion, including lymphangiogenesis and enlargement of preexisting vessels. One of the major and best-characterized growth factors involved in these processes is VEGF-C [6]. While activation of the lymphatic vasculature is therefore beneficial in the setting of chronic inflammation, there are currently no cosmetic ingredients available that specifically activate lymphatic vessels, making VEGF-C the prime candidate to address this urgent need. This is even more important that ageing results in gradual atrophy and a decrease in density and network complexity of the lymphatic system, promoting water accumulation in the tissues [7]. Thus, under-eye puffiness or mild swelling becomes more prominent as we age due to intrinsic changes in the physiology and anatomy of skin. Therefore, controlling under-eye puffiness is a daily challenge.

Besides medical treatment such as plastic surgery for the eye area, topical solutions including caffeine or retinol can be proposed. It also seems that Gentiopicroside (GP), obtained from plants belonging to gentian species that are used extensively as medicinal herbs, and which is a secoiridoid glycoside known for its wide range of pharmacological activities, can be a good candidate for reducing skin concerns in the eye area [8]. Indeed, GP has anti-inflammatory, antioxidant, antibacterial activities, and smooth muscle relaxing effect [9,10]. GP has also been documented to reduce tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels.

Given its antioxidant, anti-inflammatory and upregulatory (glutathione, superoxide dismutase and catalase) abilities via the NRF2 pathway to downregulate protein carbonylation, lipid peroxidation, NF-kB pathway, inflammatory cytokines, and vascular disorders with reduced expression of VEGF-A [11], the investigation was directed towards exploring the protective

effect of GP on the skin around the eyes. For this, an extract of *Gentiana lutea* (GlE) containing gentiopicroside (65% by dry matter) was evaluated in *in vitro*, *ex-vivo* and *in vivo* studies on various oxidative and inflammatory determinants, likely to improve the skin of the eye contours.

Materials and Methods

GIE extract

GIE extract was realized using hydro-alcoholic extraction. The intermediate extract was concentrated thanks to alcohol evaporation and then purified by chromatography to collect a fraction which is highly purified in gentiopicroside. The latter was finally stabilized in water/propanediol.

***In vitro* evaluation**

The effect of GlE on antioxidant, inflammation and angiogenesis responses was evaluated *in vitro* by RT-qPCR after treatment of Normal Human Dermal Fibroblasts (NHDF).

Cytotoxicity assessment

A cytotoxicity test was first carried out by the MTT method to determine the concentrations which will then be tested for the analysis of the expression of messenger RNA.

The extract diluted from 0.3% to 0.007% showed no cytotoxicity after 24 hours of treatment on fibroblasts.

Effect on GIE on gene expression

The dilution of the extracts retained for this investigation was 0.025%.

Normal human dermal fibroblasts (NHDF) were cultured at a concentration of 120,000 cells per well in a 24-well plate, in the presence of complete culture medium. After adhesion (24 hours after seeding), the cells were rinsed with HBSS and then treated with the product diluted

at 0.025% in the culture medium without supplements and without phenol red for 24 hours. Each condition is carried out in culture triplicate.

The cell layers of the triplicates were pooled then the mRNAs were extracted, and reverse transcription performed.

Real-time PCR (qPCR) was performed using cDNA obtained from 20 ng of mRNA in a 384-well plate in a final volume of 10 µl according to the manufacturer's recommendations. Each amplification point was performed in duplicate.

Analysis of results

Each CT obtained is then brought back to the reference gene HPRT1 to calculate the ΔCT and the normalized expression is calculated using the formula 2-ΔCT. The results were expressed as percentage of overexpression or underexpression relative to the reference condition.

Since triplicates from each cell condition were pooled prior to mRNA extraction, the results represent the mean of the 3 wells.

Effect of GlE on angiogenesis

The first assay was a co-culture assay of angiogenesis in which Human Dermal Microvascular Endothelial Cells (HMVEC-d) are co-cultured with Normal human dermal fibroblasts (NHDF) for 14 days, during which time the endothelial cells (ECs) form luminal tubules resembling capillaries *in vivo.* This co-culture was treated during 7 days with VEGF-A at 100 $ng.mL^{-1}$ or VEGF-A at 100 $ng.mL^{-1}$ and GIE at 0.3% . Samples were fixed and stained with the endothelial marker von Willebrand factor, a glycoprotein produced in endothelial cells, a marker to monitor pseudo-tubes formation. Each experimental condition was performed in triplicate and variations of pseudo-tubes area were expressed as percentage of control.

Secondly, NHDF were incubated during 48 hours with GIE at 0.3% or Phorbol 12 Myristate 13-Acetate (PMA) 1µg.mL^{-1} as a positive control. VEGF-C quantification was then realized on supernatant by sandwich ELISA with measurement at 450nm. Each condition was conducted in triplicate and variations of VEGF-C release were expressed as percentage of untreated NHDF (control).

Effect of GlE on collagen I synthesis

NHDF from eyelid of a 55 years-old female donor were treated during 72 hours with GlE 0.25% or genistein 50µM (0.001%) as a positive control. Collagen I quantification was realized on supernatant by sandwich ELISA with measurement at 450 nm and normalized by total protein content. Each condition was conducted in triplicate and results variations of synthesized collagen I were expressed as percentage of untreated NHDF (control).

<u>*Ex vivo* evaluation</u>

Effect of GIE on advanced glycation end-products

Carboxymethyl-lysine (CML), an advanced glycation end-product, and Glyoxalase-1 (Glo-1), an advanced glycation end-product detoxifying enzyme, expressions were quantified on skin explants topically treated with 0.5% (v/v) of GlE or vehicle, in basal and UVA-irradiated conditions (LED 365 nm, 2 irradiation cycles, cumulative dose of 6 J.cm^{-2}). Skin explant sections were realized and fixed. Glo-1 was detected by immunostaining using specific primary monoclonal antibody and a secondary antibody coupled to a fluorochrome (green fluorescence for Glo-1 and magenta fluorescence for CML). Nuclei were stained with DAPI. Quantification of fluorescence staining was performed (epifluorescence microscope ThermoFisher, Evos M5000) by integration of the specific fluorescence signal normalized by the surface of the

evaluation area, and then expressed as percentage of relative fluorescent unit (RFU) normalized to untreated and non-irradiated skin explants (control).

In vivo **evaluation**

An *in vivo* study was conducted with two different volunteer panels to ascertain the efficacy of GIE in improving the eye contours.

Twice applications per day on eye contour of a cream containing 0.5% of GIE on one side and a placebo cream on the other side were realized by 22 volunteers (43-64 years) with saggy upper eyelids and dark circles, and 22 volunteers (47-64 years) with eye bags and visible tear trough for 14 days.

3D image acquisition using DermaTop® and skin color measurement (Spectrophotometer®) were performed at D0, and D14. DermaTop® permits the evaluation of the upper eyelid skin aging (roughness and folds), the severity of under-eye bags (volume), and the severity of the tear trough (roughness, volume, mean depth). Spectrophotometer® permits to evaluate the color of dark circles.

Statistical analysis

Data are presented as mean ± standard deviation (SD). After having tested the normality of the data distributions, data have been analyzed by Wilcoxon signed rank test or Student's *t*-test with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

***In vitro* data**

Gene expression analysis

At 0.025%, GlE upregulated the gene expression of NFE2L2 (21%) and downregulated the expression of CXCL-8 (-20.4%). It also decreased the VEGF-A gene expression (-11%).

Effect of GlE on angiogenesis

Co-culture assay in which HMVEC-d had been co-cultured with NHDF showed that VEGF-A treatment resulted in a significant induction of tube formation.

Treatment with GIE resulted in a significant inhibition of tube formation at a GIE concentration of 0.3% ($p<0.05$), when compared with VEGF-A treated samples without GIE (Figure 1).

Insert Figure 1

GIE also induced a significant release of VEGF-C release (48%: $p<0.01$) compared to untreated fibroblasts.

Effect of GlE on collagen I synthesis

GlE treatments resulted in a significant of collagen I synthesis in eyelid derived NHDF compared to untreated NHDF by 76% ($p<0.05$) (Figure 2).

Insert Figure 2

Ex vivo **evaluation**

Effect of GIE on advanced glycation end-product

In basal condition, Glo-1 was increased with GIE 0.5% topical treatment by 19% ($p<0.01$) when compared to control (Figure 3). In UVA-stimulated conditions, Glo-1 was increased by 35% ($p<0.001$) as compared to UV-stimulated control.

Insert Figure 3

Concerning Carboxymethyl-lysine (CML), in UVA-irradiated condition, GIE 0.5% treatment significantly reduced its expression by -31% ($p<0.001$) as compared to UVA-stimulated control.

In vivo **data**

GlE cream significantly reduced the average roughness of the upper eyelid skin (-18% vs D0: $p<0.05$; -16% vs placebo: $p=0.05$) and the height of the upper eyelid skin folds after 14 days (-22% vs D0: $p<0.01$; -17% vs placebo) (Figure 4).

Insert Figure 4

The average skin relief in the tear trough area was also significantly decreased (-6% vs D0; -17% vs placebo: $p< 0.05$) after 14 days of GIE treatment.

Significant decrease in the redness of dark circles (-14% vs D0: $p<0.01$; -9% vs placebo: $p< 0.05$) and in the volume of under-eye bags (-18% vs D0; -24% vs placebo: $p< 0.05$) were also noted on the GlE-treated area.

Discussion

Maintaining a youthful appearance is a priority for many people. Systemic eye rejuvenation is sought more frequently and at a younger age than other treatments. Major concerns around the eye area are periorbital hyperpigmentation, puffiness, sagging eyelids and wrinkles. Periorbital hyperpigmentation (dark circles around and under the eye and upper eyelid discoloration) is a frequent cosmetic problem, with no gold standard treatment option available. The aim of this investigation was to evaluate whether a Gentiopicroside-rich *Gentiana lutea* extract (GIE) may modulate periorbital skin health, repair, and renew skin's appearance.

Gentiana lutea is a well-known species with a long history of use as herbal medicine in many countries, especially for its anti-inflammatory and antioxidative properties. Indeed, the dried underground parts of yellow gentian (i.e., roots and rhizome) are rich in secondary metabolites such as secoiridoids, the classes of secoiridoids comprising gentiopicroside, considered to be one of the main components responsible for bioactivity [12].

Our results shown that GIE has a significant effect on VEGF-C, an important mediator of lymphangiogenesis. At the same time, we noted that GIE extract reduced CXCL8/IL8. The lymphatic system plays an important role in tissue fluid homeostasis, and immune surveillance. Indeed, lymphatic vessels are heavily involved in regulating the inflammatory response by draining extravasated fluid, antigens, and inflammatory mediators [13]. It is well known that ageing induces in gradual atrophy and a decrease in density and network complexity of the lymphatic system, promoting water accumulation in the tissues [14]. One can put forward the hypothesis that GIE, through the activation of the VEGF-C/VEGFR-3 signaling pathway and the inhibition of the production of chemotactic inflammatory mediators, may decrease eyelid edema and puffiness.

Edema can be also linked to an increase in capillary permeability, under VEGF-A control [15]. VEGF-A regulates angiogenesis and vascular permeability by activating 2 receptors, VEGFR-

1 and VEGFR-2 expressed by endothelial cells which are known to form tube-like structures leading to neovascularization. Overproduction of VEGF-A can be induced by AGEs, a group of modified proteins and/or lipids, which also up-regulate proinflammatory cytokines such as IL-6 via RAGE-NF-Kappa B pathway activation. It is also shown that AGEs decrease collagen I levels in fibroblasts, through the p-p38 MAPK and NF-κB-p-p65 pathways activation, thereby regulating collagen metabolism, although other pathways may also participate [17]. It is also well described that an increase in the susceptibility towards enzymatic degradation is associated with glycation of elastin and structural alterations in the protein, leading to an overall reduction of tissue elastin amounts. All these phenomena induce an increase in stiffness and loss of cutaneous elasticity, and therefore skin sagging and wrinkling [18].

As shown in Figure 1, treatment with GIE resulted in a significant inhibition of tube formation at a GIE concentration of 0.3% ($p<0.05$). A significant of collagen I synthesis (76%: $p<0.05$) in eyelid derived NHDF after GIE treatment was also observed when compared to untreated NHDF (Figure 2). It is important to note that this increase is higher than those observed after a genistein treatment, known for its anti-ageing effect.

In addition to have deleterious effect on collagen and VEGF-A, AGEs increase reactive oxygen species formation and impair antioxidant systems. Nevertheless, it is important to note that the formation of some AGEs can be induced *per se* under oxidative conditions [19]. Among these AGEs is the glycoxidation product, N^{ϵ}-(carboxymethyl)lysine (CML), which is the only (with pentosidine) chemically characterized AGEs known to accumulate in protein with age [20], and which can be also derived from PUFA during lipid peroxidation reactions [21]. It can be accumulated in tissues such as skin, heart, arteries, and intervertebral disks. In skin, glycation of collagen I induces the synthesis of CML in dermal compartments and epidermal compartments and, consequently, an aging phenotype consisting of poor stratification of epidermal layers and vacuolization of keratinocyte cytoplasm [22]. To limit AGEs

accumulation in tissues, detoxification mechanisms eliminate AGE precursors which involves the glyoxalase enzymatic system, including Glo-1. With aging, Glo-1 mRNA and protein expression and enzymatic activity decrease in the brain and red blood cells but increase in other tissues such as the skin [23]. Interestingly, Glo-1 restoration is currently under investigation for the prevention of aging-related disorders. Our results showed that topical treatment of GIE extract increased Glo-1 by 19% ($p<0.01$) when compared to control (Figure 3). At the same time, the expression of CML was significantly reduced. GlE also upregulated the gene expression of NFE2L2 (21%) and downregulated the expression of CXCL-8.

Our clinical evaluation of an eye cream containing GIE extract compared to placebo showed systemic improvements in periorbital skin condition after 14 days (Figure 4). This test was highly rated by subjects for efficacy and attributes of the product, which was well tolerated.

Conclusion

The eyes and periorbital region are critical for emotive display and play a key role in social interactions. This region includes the upper and lower eyelids, brow-lid complex, and lid-cheek complex. Perturbances in this area can lead to a prematurely aged appearance and patients complain of emotive misinterpretation. By acting on AGEs pathway and VEFG-C, which specifically activate lymphatic vessels, GIE achieved a systemic skin rejuvenation by improving appearance of periorbital hyperpigmentation, puffiness, sagging and wrinkled eyelids and tear trough severity after 14 days of application. Although results of this integrate study were satisfactory in both efficacy and subject satisfaction, it would be interesting to evaluate the efficacy of GIE on periorbital skin microbiota, antibacterial gentiopicroside property being well established.

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Conflict of Interest Statement. NONE.

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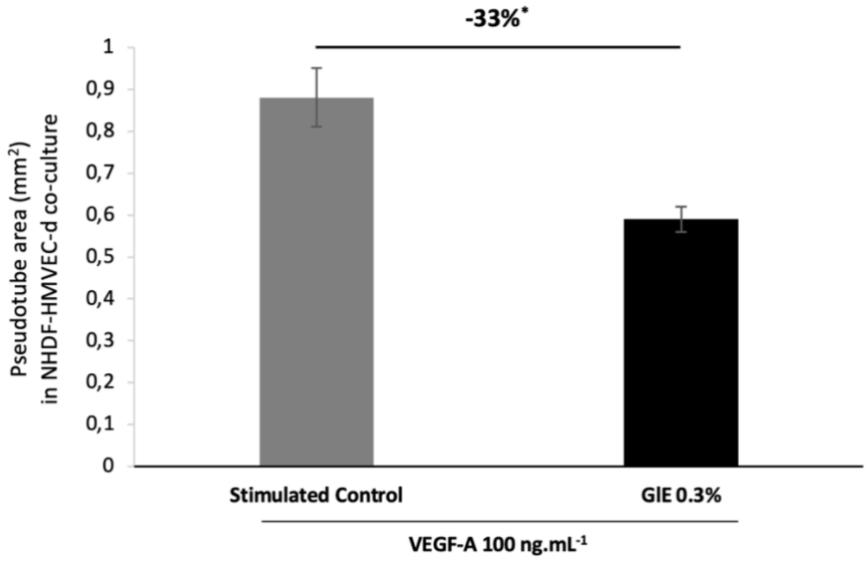

Figure 1: Pseudo tubes formation following treatment with the pro-angiogenic VEGF-A and with GIE. * $p<0.05$ vs VEGF-stimulated control.

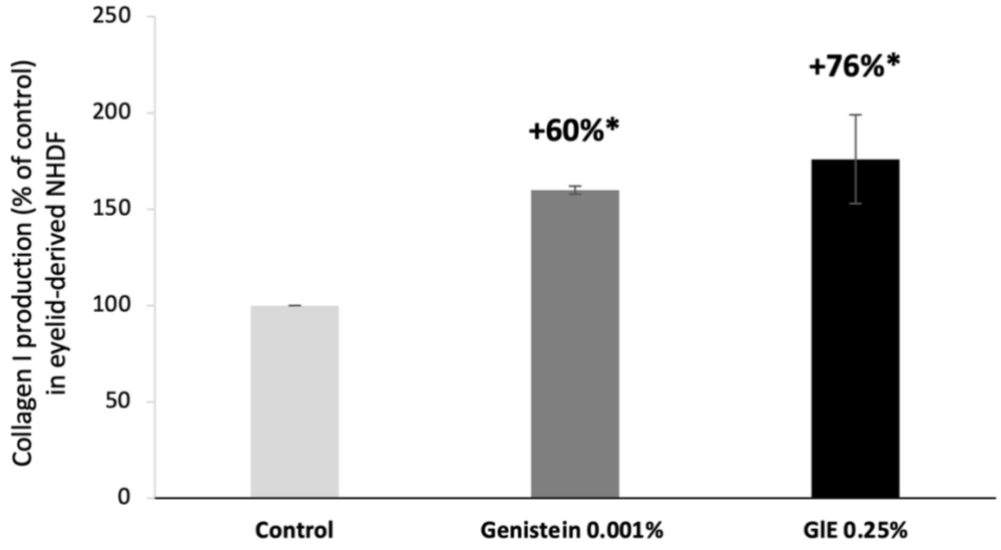

Figure 2: Collagen I synthesis by eyelid derived NHDF following treatment with genistein (positive control) or with GIE. * $p<0.05$ vs control.

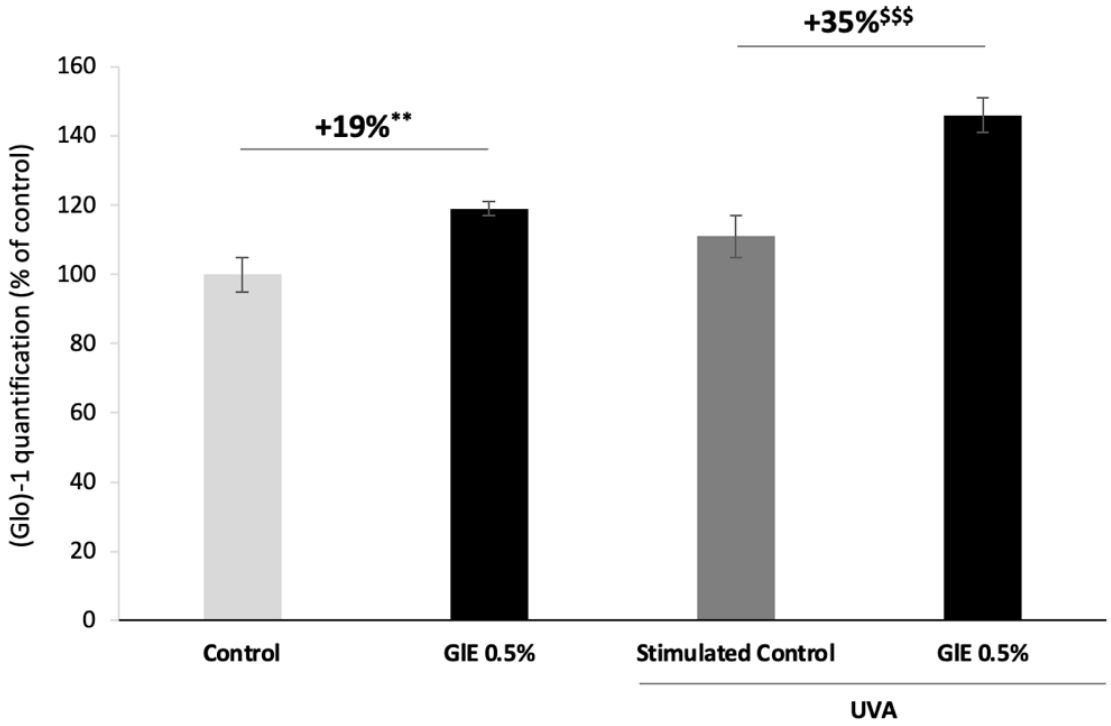

Figure 3: Glyoxalase-1 (Glo-1) quantification with GIE. ** p<0.01 vs control; $$$ p<0.001 vs UVA-stimulated control.

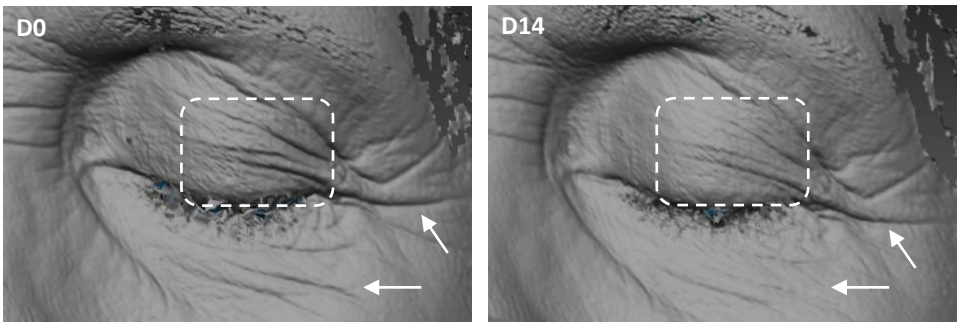

Figure 4: Illustrative 3D pictures performed with DermaTop® showing the appearance of the upper part of the eye contour at D0 and after 14 days of treatment with GlE cream. Dotted square: illustration of the improvement of the upper eyelid folds.

White arrow: illustration of the improvement of periocular wrinkles and fine lines.