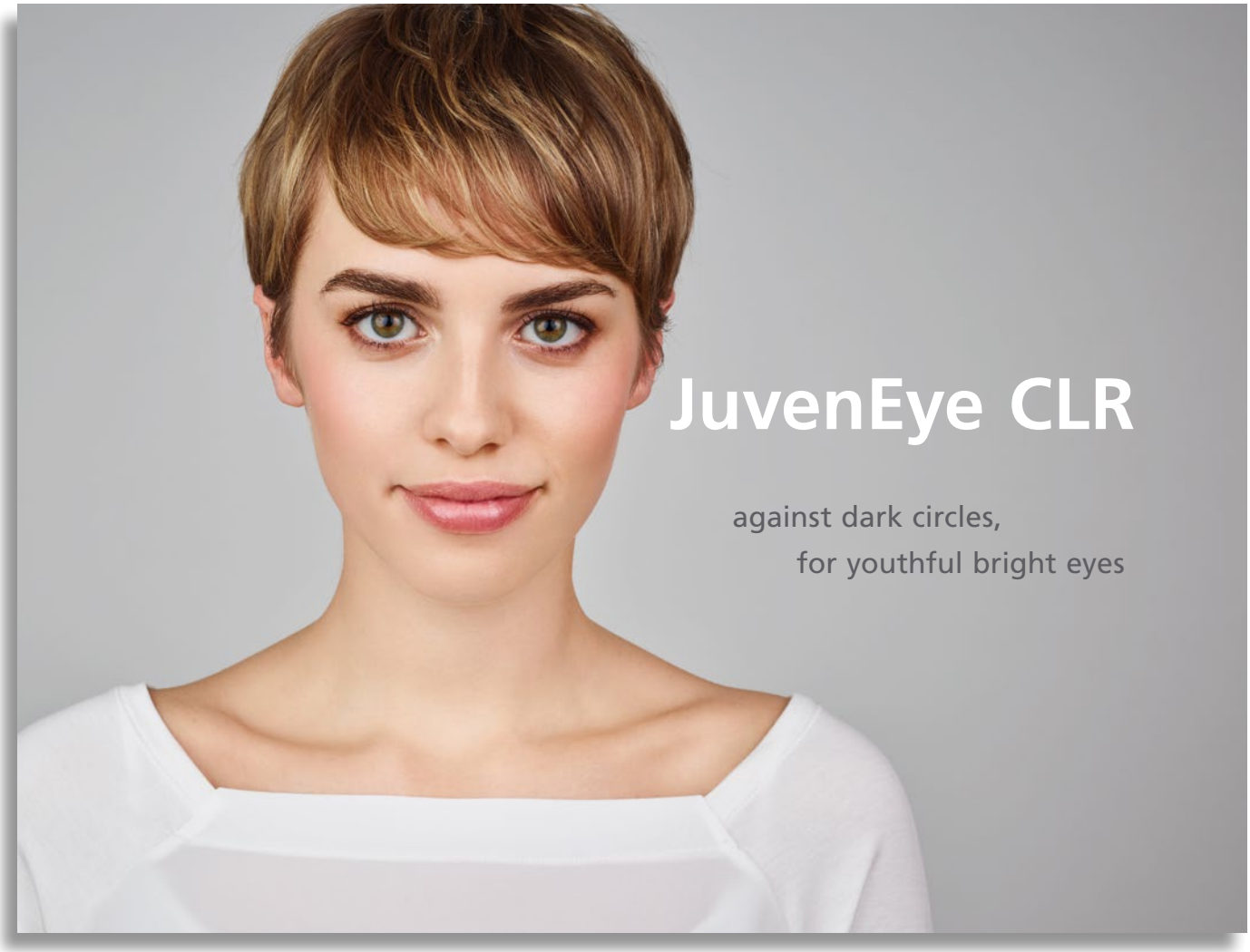


JuvenEye CLR – against dark circles, for youthful bright eyes

Introduction

Dark circles under the eyes are a common phenomenon. They make people look tired and older, and can have a significant impact on quality of life. Men and women are equally affected and, although aging plays a causative role, dark circles are seen in people of all ages.

The pathophysiology of dark circles is extremely multifactorial, with different physiological processes being intertwined with each other. In reducing the appearance of dark circles it is therefore essential to find the common causes for the different processes which lead to the formation of dark circles. Aging-related factors are important and should be addressed, but the processes behind the daily fluctuations of dark circles are at least as important. The appearance of dark circles can change from day to day.

Skin translucency

The skin under and around the eyes is particularly thin. The aging processes, both biological and accelerated by sunlight, play an important negative role in the further thinning of the skin, where collagens, etc. are broken down and the subcutis is destroyed altogether.

JuvenEye CLR

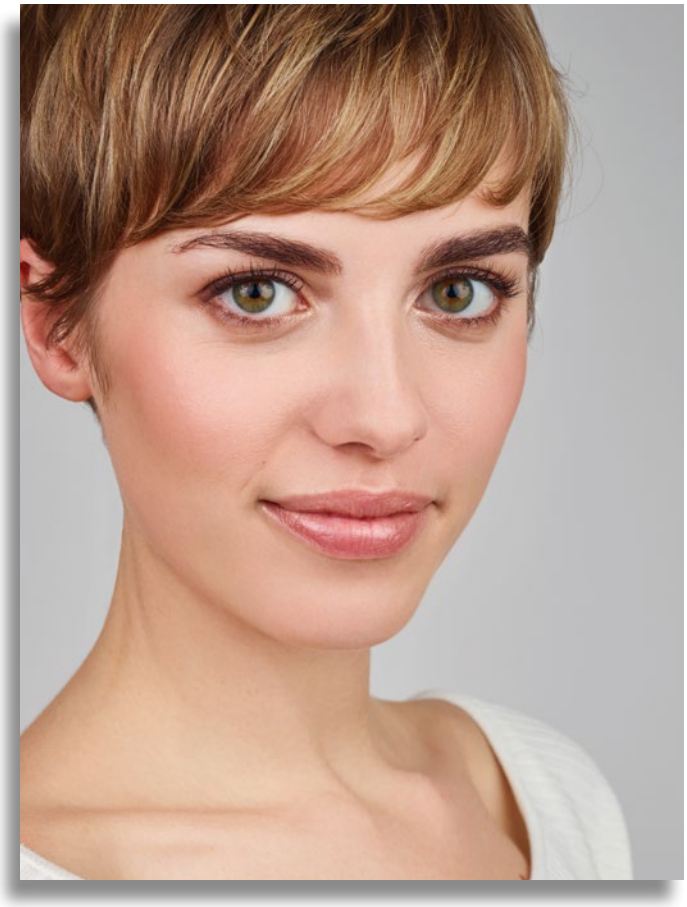
Dosage: 3.0–5.0%
pH range: 4.5–6.5
INCI Name: Hieracium Pilosella (Hawkweed) Extract, Bellis Perennis (Daisy) Flower Extract

Application

- Skin regeneration
- Skin lightening

Summary

Based on a synergistic combination of extracts of *Hieracium pilosella* and the flowers of *Bellis perennis*, **JuvenEye CLR** addresses the most important aspects in the formation and maintenance of dark circles. JuvenEye CLR induces the production of heme oxygenase and VEGF-C, activates autophagy and increases cellular functionality and energy. It also reduces melanin synthesis. The appearance of dark circles, their color and surface area are reduced.



Due to the decreasing thickness of the skin, skin becomes more translucent. This is a particular problem for the skin under the eyes, as here we find a vast network of capillary blood vessels, lymph vessels and muscles just below the surface of the skin. As a consequence, thinning of skin leads to a darkened appearance, a factor that plays an important role in the appearance of dark circles.

Skin laxity

During aging, skin becomes lax, and gravity will pull the skin of the lower eyelids down, leading to a shadowing effect, another contributor to the appearance of dark circles. Additionally, due to the increase in skin laxity, skin becomes stretched and therefore even thinner and more translucent.

Pigmentation

Damage caused by UV light can lead to hyperpigmentation. Especially people with a higher skin phototype often show a phenomenon called postinflammatory hyperpigmentation. This can play a prominent role in the appearance of dark circles.

The hyperpigmentary factor in the appearance of dark circles is not just an epidermal phenomenon. Dermal melanin deposition, where melanin-containing melanosomes have been engulfed in macrophages, resulting in the formation of so-called melanophages, has been described to play an important role in the appearance of dark circles.

Cutaneous lymphatic system

The most important function of the lymph vessels is to maintain a balance in fluid, macromolecules and oncotic pressure in the interstitial areas (extracellular space) of our body. They drain excess tissue fluid back to the blood circulation. Macromolecules and cells can directly enter the lymphatic vessels.

The lymphatic vessels become reduced in number and increasingly hyperpermeable during the aging process as well as consequential to inflammatory processes. This results in edema of the lower eyelid, an accumulation of fluid. This fluid often takes on a purplish color and can significantly influence the color of skin under the eyes.

As mentioned above, another important factor in dark circles that is specifically related to aging is dermal melanin deposition in so-called melanophages. The cutaneous lymph vessels are extremely important in trafficking macrophages out of the skin. Melanophages are macrophages containing melanin, another illustration of the importance of the cutaneous lymphatic system in the appearance of dark circles.

Hemocongestion

Blood flow in the under-eye area is sluggish and slow. Hemocongestion, where blood flow is reduced to zero, is a frequent phenomenon in skin under the eyes. Oxygenated hemoglobin has a reddish color and produces a pinkish tint in the skin. In contrast, deoxygenated hemoglobin has a purplish color and produces a tint which is more bluish. Hemocongestion is associated with a large presence of deoxygenated hemoglobin and strongly contributes to the appearance of dark circles.

Downstream of hemocongestion: heme

Consequential to hemocongestion, but also to inflammatory and aging processes, vascular permeability is increased. In the interstitial area in the dermis the leaked red blood cells burst, releasing hemoglobin. The hemoglobin rapidly releases its heme group. The color of heme is very dark and can significantly contribute to the appearance of dark circles. Additionally, heme is a molecule which can induce a multitude of deleterious reactions in the skin, many of which are relevant for the formation and maintenance of dark circles.

Heme can cause cell damage, which is especially relevant for epithelial cells of both the cutaneous blood microvasculature and the lymph vessels. Heme induces oxidative stress, the breakdown of the extracellular matrix in the dermis (collagens, elastin, etc.) and stands at the beginning of inflammatory processes, supporting the pathophysiology of the formation of dark circles.

The inflammatory processes initiated by heme result in a further increase in the permeability of both blood and lymph vessels. Heme is therefore an essential factor in fighting dark circles, both of the aging-related and the acute type, the one which can change in appearance from day to day.

Approaches toward the reduction of dark circles

To successfully reduce dark circles, it is important to focus on physiological events which stand at the crossroads of biological processes, inducing the formation of dark circles.

Heme is an important factor, as it has a dark color in itself and induces multiple cellular processes that are important in both the formation of dark circles and the vicious circle of events which seem to worsen and maintain this phenomenon. Increasing lymphatic drainage is another essential issue. Edema is reduced, and hemoglobin, heme as well as macrophages are removed. Additionally, melanin synthesis should be addressed.

Efficacy studies – *in vitro* assays

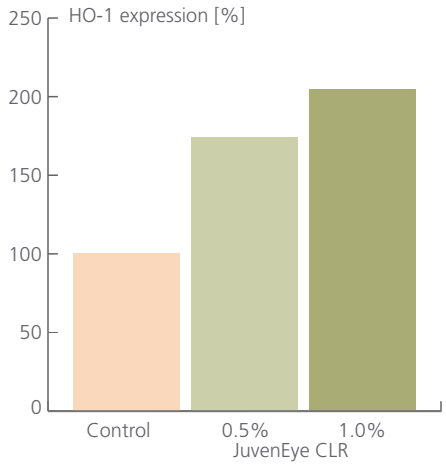


Fig. 1: JuvenEye CLR promotes the expression of HO-1 in keratinocytes

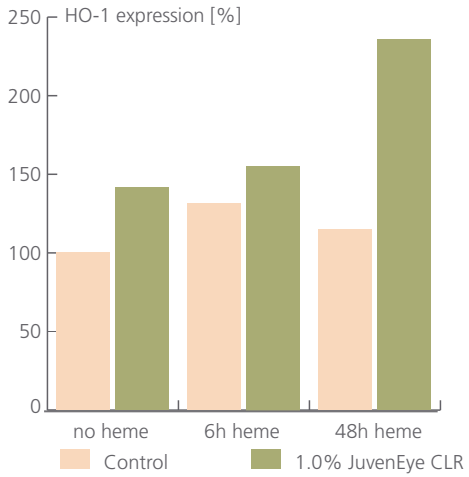


Fig. 2: JuvenEye CLR promotes the expression of HO-1, despite prolonged exposure to heme

Heme Oxygenase (HO-1)

Heme is broken down by Heme Oxygenase (HO-1). HO-1 is an enzyme that is strongly involved in the skin's defense mechanisms against oxidative stress. HO-1 is a phase II detoxifying enzyme. Heme is broken down by HO-1 to obtain biliverdin, and biliverdin can be further broken down by biliverdin reductase to bilirubin. Heme is dark-colored, biliverdin is rather greenish, and bilirubin is yellowish.

HO-1 is inducible and produced in keratinocytes. Inducing HO-1 in keratinocytes is therefore an interesting approach toward accelerating the breakdown of heme. On top of the above-mentioned effects on skin color, the breakdown of heme is of great importance in stopping the progression of the deleterious processes induced by heme, as described above.

Method

Human keratinocytes (HaCaT) were treated with the test compounds for 48 hours. The determination of HO-1 was performed by ELISA (R&D Systems, Inc.; KCB3776). Relative fluorescence units were determined at 540 nm (ex.)/600 nm (em.). Non-treated cells are set at 100%.

Results

At different concentrations, JuvenEye CLR strongly promotes the production of HO-1 in keratinocytes (HaCaT) (Fig. 1). This indicates that JuvenEye CLR promotes heme degradation and supports one of the most important processes which counteract the formation of dark circles.

Heme and HO-1 expression

Heme is pro-oxidative and pro-inflammatory. The presence of heme, therefore, can have a negative impact on skin cell functionality. As keratinocytes have the ability to produce HO-1 and can be exposed to heme, it is important to verify that the effect of heme on HO-1 production in keratinocytes is not negatively influenced.

Method

Human keratinocytes (HaCaT) were pretreated with JuvenEye CLR for 24 hours. Heme (5 µM) was added for different periods of time. The determination of HO-1 was performed by ELISA (R&D Systems, Inc.; KCB3776). Relative fluorescence units were determined at 540 nm (ex.)/600 nm (em.). Non-treated cells are set at 100%.

Results

Heme treatment of keratinocytes (HaCaT) cells at first led to an increase of HO-1 production, but prolonged exposure (48 h) to heme led to a clear reduction of HO-1 production (Fig. 2). As heme is proinflammatory and pro-oxidative, this is likely a result of the prolonged stress the cells are exposed to. JuvenEye CLR allows the cells to deal with the presence of heme, leading to an increased production of HO-1 after prolonged exposure to heme. This effect could not be observed in the control cells. The experiment mimicked the *in vivo* situation in dark circles, where prolonged heme exposure can have deleterious effect on the skin cells. JuvenEye CLR showed that it can compensate effectively for these effects.

Cellular energy and metabolic turnover

The functionality of the skin cells, in particular keratinocytes from the epidermis, suffers with age and under inflammatory circumstances. Keratinocytes play an essential role in cell signaling. They are at the basis of cell signaling toward the dermal compartment, the area in the skin where we find the extracellular matrix (collagens, etc.) and blood and lymph vessels. Loss in keratinocyte functionality can have a negative impact on the structures in the dermal compartment and should therefore be addressed.

Method

Human keratinocytes (HaCaT) were pretreated with JuvenEye CLR for 24 hours. Determination of ATP was performed by luminescent adenosine triphosphate detection assay (Packard Instrument Company, Inc.; 6016541). The luminescence was determined in a luminescence reader (Spectramax Paradigm, Molecular Devices, LLC.). The metabolic activity was measured with tetrazolium salt (MTT) assay. The absorbance was determined in a microplate reader (Spectramax Paradigm, Molecular Devices, LLC.) at 570 nm. Non-treated cells served as controls and were set at 100%.

Results

JuvenEye CLR at both 0.5% and 1.0% led to an increase of metabolic activity and ATP (energy) production in keratinocytes (HaCaT) (Fig. 3). JuvenEye CLR therefore showed to have a positive influence on these cells, increasing their functionality and health. At least part of the results obtained here can be attributed to the influence of JuvenEye CLR on autophagy, as described below. One of the key features in dark circles is cellular stress, which can have a significant impact on cell functionality. The results from this experiment show that JuvenEye CLR supports the skin cells in compensating for this stress.

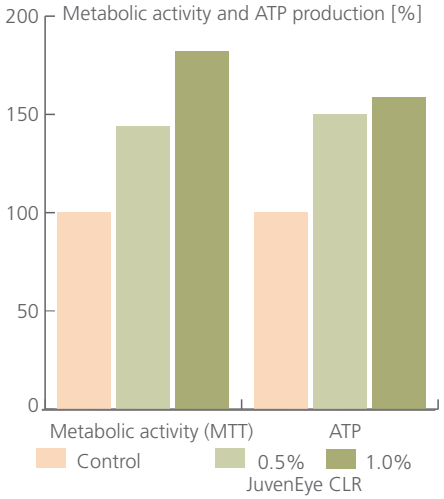


Fig. 3: JuvenEye CLR increases both metabolic activity and energy production in keratinocytes

Autophagy

Autophagy is a process by which cells can recycle old dysfunctional organelles and protein aggregates. The accumulation of such "cellular waste" is an important feature in cellular aging. Induction of autophagy, therefore, is a sensible anti-aging approach which increases cellular longevity and functionality.

Autophagy plays another important role in the context of dark circles. It is a process with which keratinocytes degrade melanin-containing melanosomes. In this context autophagy is described as an important factor in regulating skin color, and induction of autophagy is therefore an effective approach to lighten skin.

Method

Human keratinocytes (HaCaT) were pretreated with JuvenEye CLR for 24 hours. Cells were treated with 2 µM Chloroquine (inhibition of autophagy flux). Cells were incubated for further 48 hours w/wo JuvenEye CLR. Determination of LC3B was performed by ELISA using LC3B (D11) XP® mA (New England Biolabs, Inc.). The detection antibody was an anti-IgG (H+L), F (ab')2 fragment (Alexa®488) (New England Biolabs, Inc.). The fluorescence was measured at 485 nm (ex.) and 535 nm (em.) in a fluorescence reader (Spectramax Paradigm, Molecular Devices, LLC.). Non-treated cells served as controls and were set at 100%.

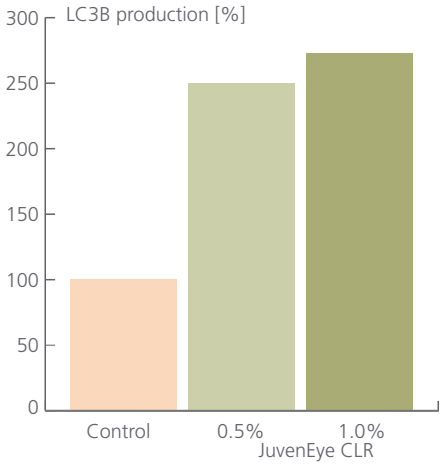


Fig. 4: JuvenEye CLR promotes autophagy

Results

JuvenEye CLR at both 0.5% and 1.0% strongly induced autophagy in keratinocytes (HaCaT) (Fig. 4). This indicates that it strongly supports the cellular anti-aging processes and can increase cellular functionality (as demonstrated above). JuvenEye CLR supports the cellular processes in breaking down melanosomes, leading to skin-lightening activities.

Melanogenesis under the influence of heme and UV radiation

Pigmentation – both aging-related and consequential to inflammatory processes – plays an important role in the appearance of dark circles. Heme is proinflammatory and pro-oxidative. It plays an important role as a "motor" behind the formation of dark circles. It is an inducer of melanogenesis. UV radiation is an important inducer of melanogenesis as well. Like heme, UV light induces inflammatory and oxidative processes in the skin. It is therefore of interest to study whether the treatment with heme and heme together with UV radiation, has an influence on melanin synthesis, and if JuvenEye CLR can reduce melanin synthesis induced by heme and UV light.

Method

Week 1: Human epidermal equivalents with melanocytes (epiCS-M, CellSystems GmbH) were treated for one week with 10 µM heme on the basolateral side. Some of the skin models were irradiated with 0.3 J/cm² UVA + 0.03 J/cm² UVB once daily. Week 2: Heme treatment and irradiation was stopped, and the models were treated with either water (control) or 3% JuvenEye CLR (in water) for 7 days. Melanin content of the skin models was extracted and quantified by photometry. Non-treated skin models served as controls and were set at 0%.

Results

Human epidermal equivalents with melanocytes, exposed to heme, clearly show an increased production of melanin. In this setting, the treatment with JuvenEye CLR shows a clear reduction of melanin production (Fig. 5). Exposure of these epidermal skin equivalents to both heme and UV radiation shows a further increase in melanin production. Here, the treatment with JuvenEye CLR shows a greater reduction of melanin production than with the exposure to heme only. These results clearly demonstrate that JuvenEye CLR reduces melanin production under circumstances which are extremely relevant to the formation and maintenance of dark circles.

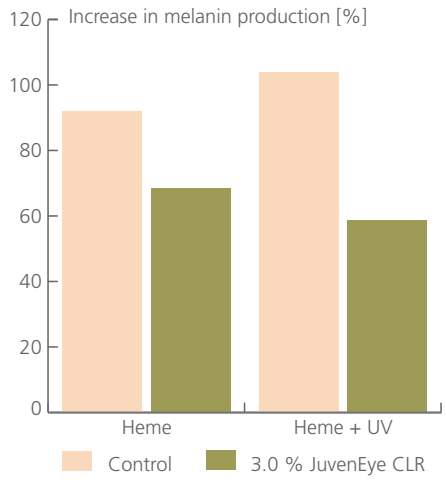


Fig. 5: JuvenEye CLR reduces melanin production in epidermal skin models containing melanocytes and stressed by heme and UV radiation

VEGF-C and lymphatic endothelial cells

VEGF-C (Vascular Endothelial Growth Factor-C) is a growth factor which promotes the production of functional lymphatic vessels. These are of eminent importance in the etiology of dark circles, as described above. Inducing the production of VEGF-C is therefore extremely important in fighting dark circles. In the eye area fluid can accumulate relatively easily. This fluid contains cells, ions and macromolecules (proteins specifically). The ions lead to osmotic pressure, whereas the proteins cause a phenomenon called oncotic pressure, a type of osmotic pressure. In an experiment that determines the effect of an active ingredient of VEGF-C expression, osmotic pressure should therefore be taken into account. As keratinocytes play an essential role in cellular communication, including toward the lymphatic endothelial cells, it is of particular interest to analyze the effect of JuvenEye CLR in an experimental design where the effect of keratinocyte communication to lymphatic endothelial cells, relevant to VEGF-C expression, is determined.

Method

Human dermal lymphatic endothelial cells (HDLEC) were pretreated with JuvenEye CLR for 24 hours. Osmolarity of the medium was adjusted by NaCl solution. The determination of VEGF-C was performed by ELISA (R&D Systems, Inc.; #DVEC00). Absorbance was measured at 450 nm/540 nm. Non-treated, non-damaged cells served as controls and were set at 0%.

Results

An increase in osmolarity led to an increase in VEGF-C expression by lymphatic endothelial cells. The treatment with JuvenEye CLR under hyperosmotic pressure led to an increase in expression of VEGF-C (Fig. 6). The positive and relevant influence on new lymph vessel production by JuvenEye CLR is thus confirmed.

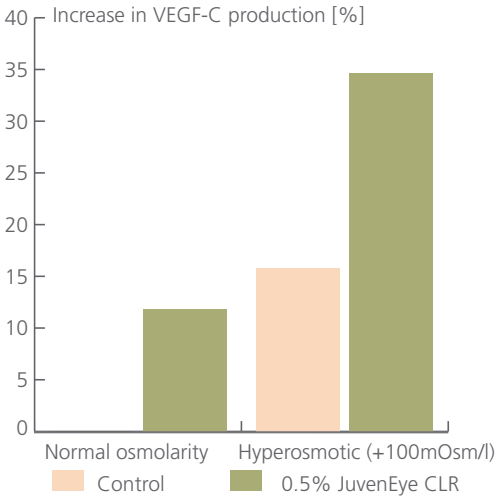


Fig. 6: JuvenEye CLR increases VEGF-C production in lymphatic endothelial cells

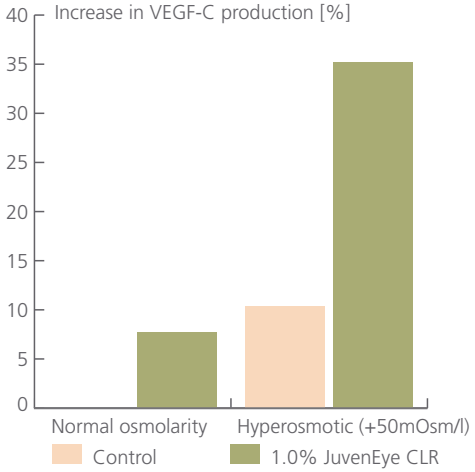


Fig. 7: JuvenEye CLR increases VEGF-C production in lymphatic endothelial cells

VEGF-C, lymphatic endothelial cells and keratinocytes

Method

In an *in vitro* situation, keratinocytes release their mediators into the cell culture medium. This medium can be transferred to lymphatic endothelial cells, and their ability to express VEGF-C can be determined. Human keratinocytes (HaCaT) were pretreated with JuvenEye CLR for 24 hours. Osmolarity of the medium was adjusted by NaCl solution, and cells were incubated for a further 24 hours.

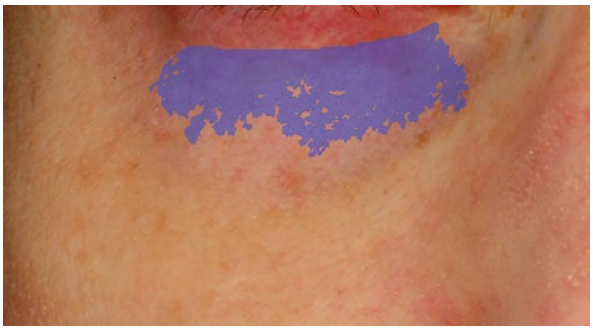
The medium was transferred to the human dermal lymphatic endothelial cells (HDLEC), which were further incubated for 24 hours. The determination of VEGF-C was performed by ELISA (R&D Systems, Inc.; #DVEC00). Absorbance was measured at 450 nm/540 nm. Non-treated, non-damaged cells served as controls and were set at 0%.

Results

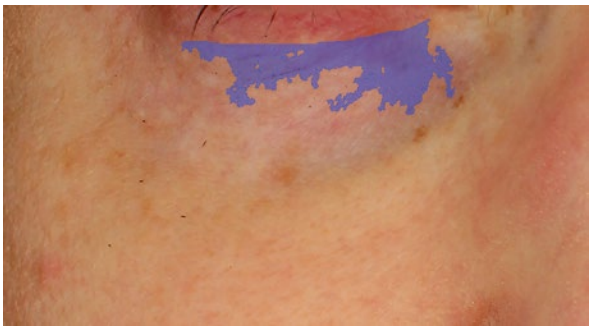
Osmotic pressure is stressful for keratinocytes. The cultivation of lymphatic endothelial cells in a medium which originates from hyperosmotically stressed keratinocytes led to a slight increase in VEGF-C (Fig. 7). In an identical setting that also included the treatment of the keratinocytes by JuvenEye CLR, a much clearer increase in VEGF-C production by lymphatic endothelial cells could be observed.

Cultivation of the lymphatic endothelial vessels in a medium originating from the treatment of keratinocytes at normal osmolarity and in the presence of JuvenEye CLR, also increased VEGF-C production, albeit slightly. These results indicate that JuvenEye CLR supports the formation of new and functional lymph vessels. JuvenEye CLR thus plays an important role in the promotion of drainage of excess fluid, inflammatory cells and heme from the interstitial area in the dermis, an activity which is essential in fighting dark circles.

Efficacy studies – *in vivo* assays



Volunteer 1: t=0



Volunteer 1: t=56 days

Fig. 8: 3% JuvenEye CLR clearly reduces the surface area of dark circles after 56 days of use

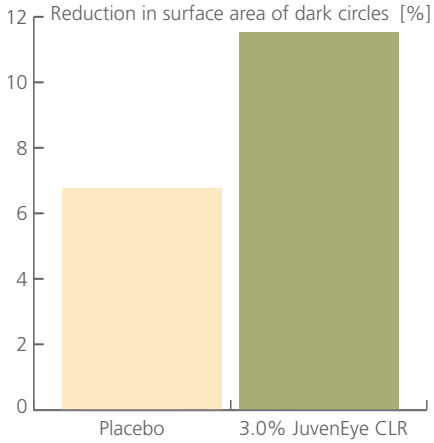


Fig. 9: JuvenEye CLR leads to a stronger reduction of the surface area of dark circles than placebo

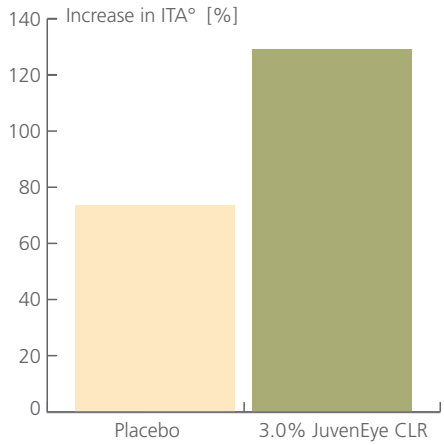


Fig. 10: JuvenEye CLR leads to a stronger reduction of the color of dark circles than placebo

Reduction of dark circles

In order to assess whether JuvenEye CLR reduces dark circles, a study was performed where 12 volunteers applied cosmetic formulations, one containing 3% JuvenEye CLR and a corresponding placebo, for 56 days, twice daily on each eye area. At the beginning of the study and after 56 days of applying the cosmetic products, standardized photographs were taken with a VISIA® device (Canfield Scientific, Inc.). This device allows for detailed imaging in a standardized environment, preventing external light influences. This makes possible a comparison of different pictures of the same person taken at different times.

The standardized photographs were then digitally analyzed by Newtone Technologies (Lyon, France). By making use of the combined technologies offered by Canfield and Newtone, conclusions can be drawn on the effect of a cosmetic product on dark circles.

Through the digital analysis of the photographs, important parameters relevant to dark circles are generated, for instance the ITA° (Individual Typological Angle). The ITA° is a relevant parameter for darkness of skin. Overall, a reduction of dark circles is obtained when ITA° increases in value.

An essential feature in the reduction of dark circles is their surface area, whose reduction is extraordinarily important for reducing their visibility.

Individual results show that the application of 3% JuvenEye CLR for 56 days clearly led to a reduction of the surface area of dark circles (Fig. 8).

An analysis of the effects obtained with 3% JuvenEye CLR and placebo formulation, now with a focus on the actual surface area of the dark circles on all individuals (Fig. 9), clearly shows that 3% JuvenEye CLR reduces the surface area of the dark circles more effectively than placebo.

To further underline the effects of JuvenEye CLR on dark circles, an additional analysis was performed on the parameters relevant to their color and visibility. The application of a formulation containing 3% JuvenEye CLR led to an increase in value of the parameter ITA°, that was clearly larger than that obtained with placebo (Fig. 10).

Legal disclaimer

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Berlin, 04.2017

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