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Crocus bulb extract prompts epidermis/dermis crosstalk

ABSTRACT

The epidermis communicates with the subjacent dermis via signalling molecules. This crosstalk is particularly important for the regulation of complex events such as wound healing, but also for continuous tissue repair and regeneration. The messenger molecules involved are mainly cytokines and growth factors. An imbalance in the synthesis and release of these signals as it is observed in elderly skin may lead to a reduced biosynthesis of important extracellular-matrix (ECM) proteins such as collagen and elastin. Here we present a strategy to activate matrix protein production by stimulating growth factor synthesis and release by keratinocytes, which can easily be reached by superficially applied cosmetic compounds. *In vitro* assays demonstrate that a crocus bulb extract can activate expression of elastin, lysyl oxidase-like 2 enzyme (LOXL2) and the connective tissue growth factor (CTGF). Non-invasive skin structure analysis by two-photon microscopy performed after a two-week treatment with a cream containing the crocus bulb extract demonstrates a clear augmentation of collagen and elastin.

Interactions between growth factors, skin cells and the extra-cellular matrix (ECM) are essential for tissue regeneration in wound healing[1] as well as intrinsic ageing of the skin. Wound healing is a complex process comprising different phases such as inflammation, proliferation and remodelling, all of which require growth factors to regulate the fine balance between the synthesis of extracellular matrix and its degradation by proteases.[2] Intrinsic skin ageing is accompanied by an accumulation of reactive oxygen species (ROS) due to an impaired mitochondrial oxidative metabolism. Enhanced ROS formation will affect cell signalling pathways which lead to an increased break down of extracellular matrix components.[3] The thinning and fragility of elderly skin is the result of an imbalance of degradation and regeneration of skin tissue. More recently, studies by Quan and colleagues find an additional reason for the loss of matrix structure in a decreased synthesis and release of growth factors.[4] Specifically this work suggests that a decrease in the growth factors CTGF and transforming growth factor-β (TGF-β) in aged skin and fibroblasts leads to a decrease in the expression of extracellular structural proteins. CTGF is primarily induced by TGF-β and appears to function as a down-stream mediator in the activation of extracellular matrix synthesis. Therefore one might ask: could ageing skin be rejuvenated by supplementing growth factors? The use of natural human growth factors in cosmetic products is prohibited. Biotechnologically-produced growth factors could theoretically be used for cosmetic products, but their efficacy is very limited for the following reasons: most growth factors are proteins of high molecular weight and complex conformation, which reach a limited penetration depth into the skin. Moreover, growth factors were shown to be unstable in cosmetic formulations, they lose their functionality and are quickly degraded by proteases following superficial application.

Here we describe an approach to stimulate the growth factor-dependent synthesis of matrix proteins in the dermis by improving keratinocyte-fibroblast communication. In the present study we investigated the effect of specific plant bulb extracts on human keratinocyte cell cultures for the purpose of enhancing the synthesis and release of growth factors, which stimulate fibroblast cells to synthesise extracellular matrix proteins. In a plant the bulb serves as a food reserve in order to support the newly growing plant with nutrients. We identified a bulb extract from *Crocus chrysanthus* with the ability to activate intercellular communication.

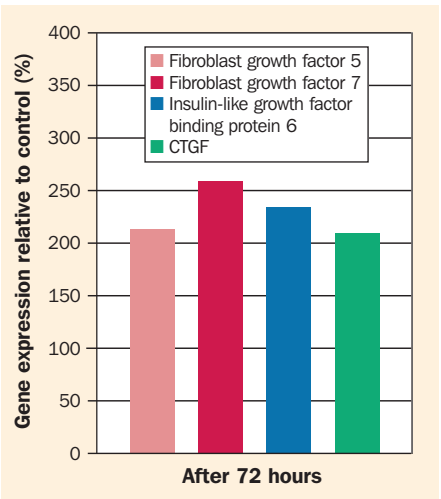


Figure 1: *Effect of an extract from* Crocus chrysanthus *bulbs on specific growth factor gene expression in keratinocytes.*

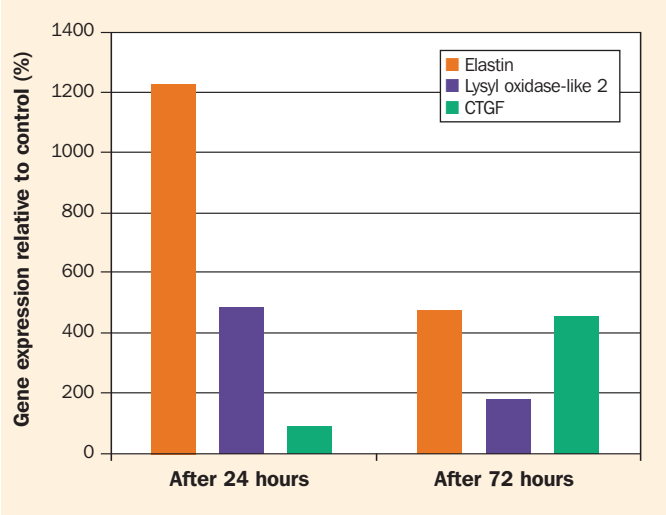
Materials and methods

Preparation of extracts from plant bulbs

Bulb extracts were prepared from species of the Amaryllidaceae, Asparagacea, Iridaceae, Liliaceae and Myrsinaceae families. A mixture containing 12% (w/w) plant bulbs and 15% (w/w) ethanol in water was incubated at 50°C for 4 hours in a DIG-MAZ 50 system (Samtech Extraktionstechnik GmbH). The extraction process was controlled by chromatographic fingerprint analysis (Waters, ACQUITY UPLC system, C18 column).

Screening assay

Primary human keratinocytes from a 50-year-old donor were cultured in an appropriate liquid medium for 24 hours. The medium was then replaced with the assay medium (Epilife medium) containing the different plant bulb extracts at non-toxic concentrations as was previously determined in a cytotoxicity assay (MTT). The keratinocytes were then incubated for 72 hours. Afterwards, the keratinocyte cell cultures were centrifuged and the cell pellets as well as the culture supernatants (keratinocyte-conditioned media) collected. The keratinocyte cells were used to analyse the expression of 30 genes coding for different growth factors and cytokines typically expressed in this cell type. The gene-expression analysis was done by quantitative PCR (LightCycler



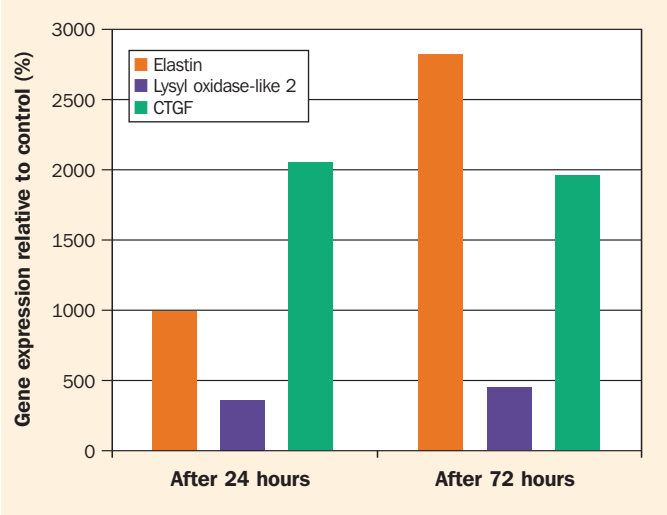


Figure 2 (left): *Effect on Elastin, LOXL2 and CTGF gene expression in fibroblasts after incubation for 24 and 72 hours with the keratinocyte-conditioned medium. The keratinocytes were treated with the crocus bulb extract. The values are compared to gene expression values of fibroblasts after incubation with the conditioned medium of untreated keratinocytes (100%).* **Figure 3:** *Effect of TGF-β on gene expression in fibroblast cells.*

system; Roche Molecular System Inc.).

The keratinocyte-conditioned media were used to treat normal human dermal fibroblasts (pool of donors over 50 years old). The fibroblasts cells were cultured for 24 hours in a standard culture medium and then for 72 hours in an assay medium (Epilife medium). The medium was then removed and replaced by either, a) the keratinocyte-conditioned medium, b) the assay medium with TGF-β (10 ng/mL), c) the assay medium containing the plant bulb extracts or d) the assay medium alone serving as control. After incubation for 24 or 72 hours, the fibroblasts were isolated and subjected to gene expression analysis of 61 genes known to play a key role in fibroblast physiology and cutaneous ageing. These experiments were performed in triplicate for verification.

Clinical study using two-photon microscopy

0.4% crocus bulb extract [DermCom/INCI name: Crocus Chrysanthus Bulb Extract (and) Acacia Senegal Gum (and) Aqua/Water] was formulated into a cream and tested in a study over a period of 4 weeks on a 53-year-old Caucasian woman. First the pre-application baseline images were characterised. Then a cream was applied twice-a-day to one inner forearm. The other forearm was treated with the corresponding placebo cream. Two-photon microscopic images of the skin were taken after two and after four weeks of treatment. Stacks of images at depths increasing up to 200 µm below the skin surface were acquired using the Olympus Fluoview 1000 MP two-photon microscopy setup customised for human skin imaging *in vivo*. Two-photon microscopic images were obtained simultaneously in two modes: auto-fluorescence (AF) and second-harmonic generation (SHG). The images reveal changes in collagen and elastin levels, which were quantified and statistically compared to the baseline and to the placebo.

Clinical anti-ageing study

A cream containing 2% crocus bulb extract was tested in a clinical study with 20 healthy volunteers, average age 47. The cream was applied twice daily for 4 weeks to the inner side of the forearm and on the crow's feet area. The other forearm and contralateral side of the face were treated with the placebo cream. Eight to twelve hours after the last product application on days 14 and 28, skin firmness and elasticity on the forearms and wrinkle depth around the eyes were measured by means of the Cutometer MPA 580, Courage & Khazaka GmbH and PRIMOS, GFMesstechnik GmbH.

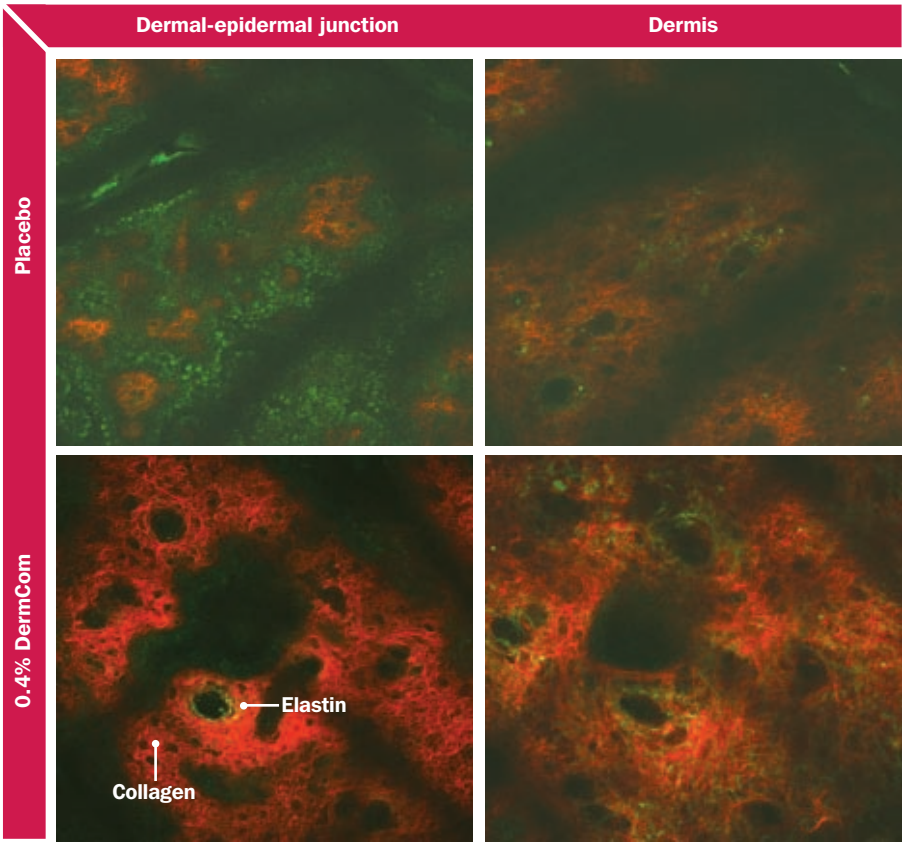


Figure 4: *Two-photon microscopic images taken at different skin depths. Skin area was treated with 0.4% DermCom in a cream. Auto-fluorescence signal shown in green, second-harmonic generation signal in red.*

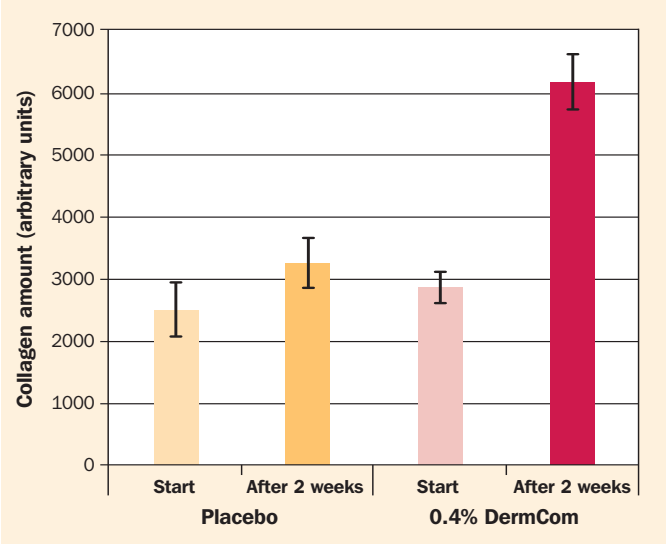


Figure 5: *Increase of collagen content in the upper dermis after two weeks of treatment with a cream containing 0.4% DermCom.*

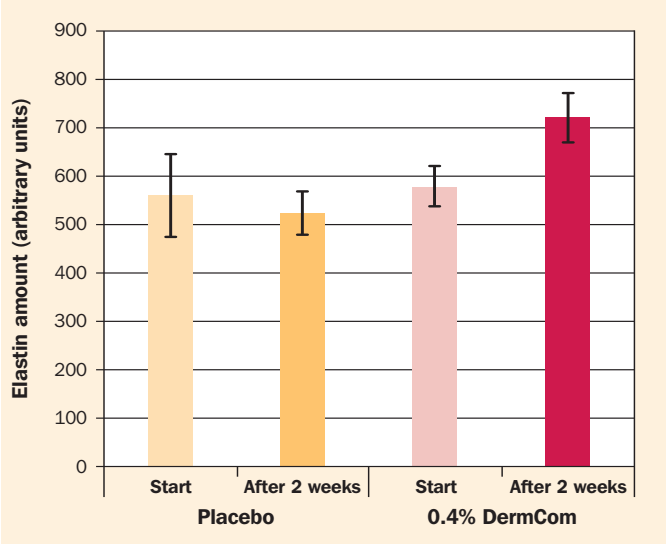


Figure 6: *Increase of elastin content in the upper dermis after two weeks of treatment with a cream containing 0.4% DermCom.*

Results and discussion

Bulb extracts from species of the Amaryllidaceae, Asparagaceae, Iridaceae, Liliaceae and Myrsinaceae families were tested in a screening assay for growth factor signalling in order to identify one that best triggers intercellular communication. Treatment of a keratinocyte cell culture with a bulb extract obtained from *Crocus chrysanthus* (Iridaceae) was shown to up-regulate the expression of genes coding for growth factors, which are known to play an important role in the epidermis/dermis crosstalk (Fig. 1). Members of the fibroblast growth factor family are involved in a variety of biological processes including cell growth, morphogenesis and tissue repair. Insulin-like growth factor binding proteins modulate the activity of growth factors.

In another set of experiments, human fibroblast cells were incubated with the conditioned medium derived from keratinocyte cultures treated with the above plant bulb extract. Figure 2 shows that this keratinocyte-conditioned medium greatly enhances the expression of genes involved in the synthesis of matrix proteins in comparison to the control culture incubated with the conditioned medium from untreated keratinocytes. Results also reveal that elastin expression increases 12-fold and LOXL2 increases 5-fold after 24 hours of incubation.

LOXL2 plays a critical role in the formation of the extracellular matrix by cross-linking elastin, which is essential for the stabilisation of this fibrous protein. It has been shown that the concentration of lysyl oxidase-like enzymes is reduced in aged skin.[5] Also, CTGF expression was found to be increased after 72 hours of incubation. The role of CTGF in adult tissue has previously been linked to

fibrosis, a group of diseases with excessive formation of fibrous connective tissue. However, Quan and co-workers showed that the down-regulation of CTGF likely mediates reduced collagen and elastin expression in aged human skin.[4] In our experiment, TGF-β was used as a positive control since it mediates the synthesis of extracellular matrix proteins. The comparison of Figure 2 and 3 illustrates that fibroblast cells treated with TGF-β exhibit a similar though stronger expression pattern compared to the cells incubated with the conditioned medium. A direct incubation of fibroblast cells with the crocus bulb extract resulted in no effect on the gene expression pattern. This indicates that the observed stimulating effect on fibroblasts depends on signalling molecules secreted by keratinocytes.

A clinical study using a two-photon microscopy was performed for the purpose of analysing the effect of a topically applied cream containing 0.4% crocus bulb extract on skin structure. In the two-photon microscopic image shown in Figure 4 the AF signal in green is generated for example by elastin fibres and compounds such as NADH and FAD. The SHG signal in red highlights collagen fibres. The results of this study demonstrate that the verum is effective as it significantly increases the amount of collagen in the papillary dermis and in the upper part of the reticular dermis (Fig. 4 lower panel) after only two weeks of treatment compared to the placebo treated skin (upper panel). Statistical analysis reveals an increase in collagen content by 115% relative to initial conditions (Fig. 5). Less pronounced but also enhanced is the increase in elastin content by 25% compared to initial conditions (Fig. 6). In both cases, the difference between the verum and placebo treated forearms was highly significant. The increase in collagen and elastin observed after two weeks continues until the end of the experiment at week four. However, the difference between placebo and verum-treated skin is less pronounced after four weeks.

Furthermore, the two-photon micrographs reveal an improvement in wrinkle width and depth. This effect is clearly seen in the upper panel of Figure 7, where wrinkles appear as black, crack-like openings that are wide at a skin depth of 12 microns and become narrower at 24 and 48 microns in depth. By contrast, images of the verum-treated skin (lower panel) show a complete absence of such micro-cracks.

These results are further supported by our second *in vivo* study on 20 volunteers, where a cream containing 2% crocus bulb extract was applied to the crow's feet area. The wrinkle depth was reduced significantly after two weeks treatment, on average by

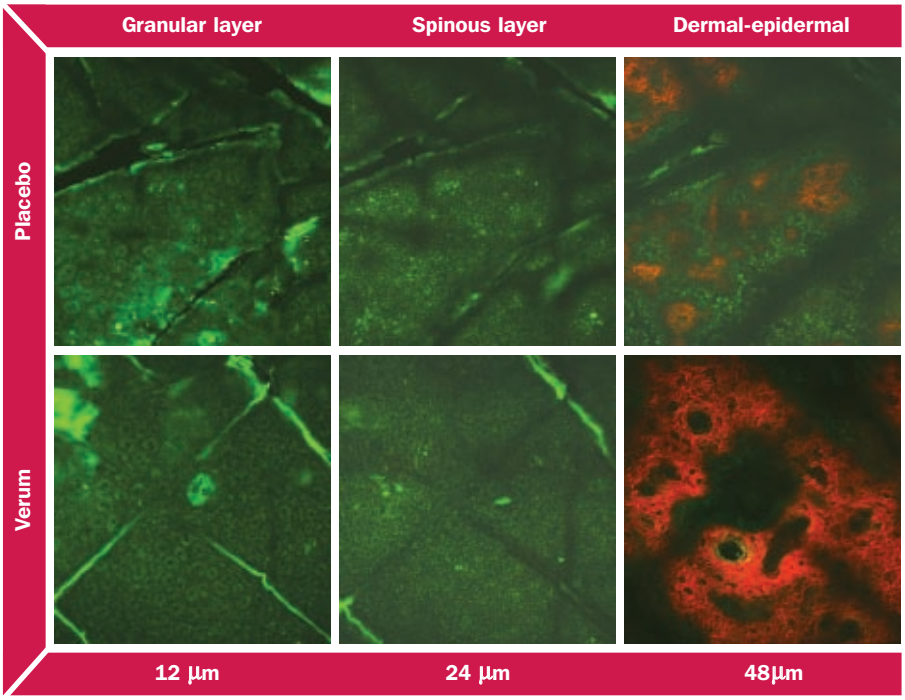


Figure 7: *Two-photon microscopic images of the epidermis after two weeks of treatment with a cream containing 0.4% DermCom.*

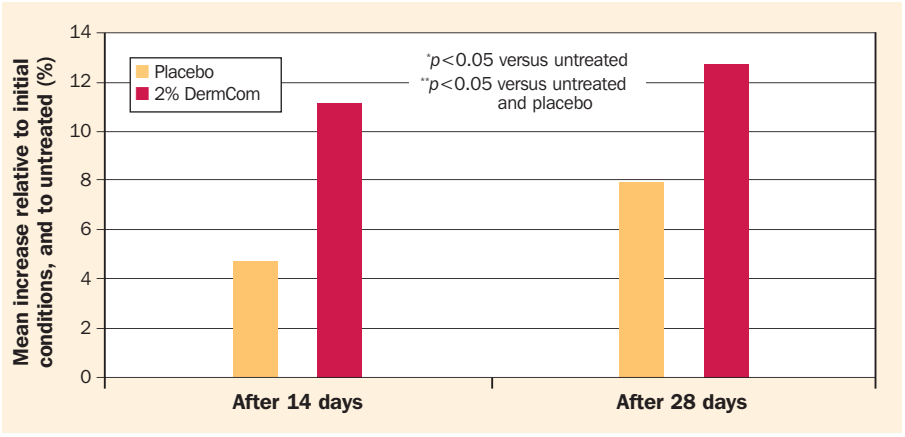


Figure 8: *Increase in skin firmness.*

9% relative to initial conditions. After four weeks of treatment on inner forearms, skin firmness was improved by 13% both compared to initial conditions and untreated skin (Fig. 8).

Conclusions

Aged skin and fibroblasts exhibit a decrease in the synthesis of growth factors. Wrinkles, which originate in the dermis are the main manifestation of skin ageing and indicate a loss in elasticity and tensile strength. The dermis is not easily reached by topically applied cosmetic actives. DermCom derived from *Crocus chrysanthus* bulbs, overcomes this problem by stimulating the keratinocytes in the top layer of the skin to synthesise and release growth factors. These act as messenger molecules to induce the production of extracellular matrix proteins in the dermis, thus improving skin firmness. PC

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