A Novel Strategy for Holistic Skin Rejuvenation

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abstract

A revolutionary anti-aging concept, senolytics, has entered the medical world. Demonstrating remarkable results in early trials, senolytics can provoke rejuvenation in various tissues by specifically eliminating senescent cells without harming healthy cells. This concept has now been adapted for the cosmetic industry, by targeting and eliminating senescent fibroblasts in aged and photo-aged skin. To optimally complete skin rejuvenation however, the eliminated fibroblasts must be replaced with new cells – a feat that can be achieved by the activation of dermal stem cells. This revolutionary idea has been realized with the innovative ingredient concept SenoCellTec™.

Introduction

The formation and accumulation of senescent cells is one of the critical hallmarks of aging [1]. Senescent cells are also called "zombie cells" since they no longer divide but are also far from being dead. As such, senescent cells continue to secrete signaling molecules that promote inflammation and further affect surrounding cells, inducing them to become senescent as well. Intriguingly, a recent publication by *Franco et al.* [2] suggests that senescent cells in the skin may even contribute to the general aging of the body. This theory underlines the importance of incorporating senolytic ingredients in cosmetics.

The senescence-associated secretory phenotype (SASP), characterized by the secretion of molecules such as inflammatory cytokines and proteases, results in the presence of high levels of the typical aging markers in the skin. In younger tissues, senescent cells are usually cleared by the immune system. In aged skin, however, with a reduced immune function, this clearance is impaired, and the senescent cells accumulate. The new concept "senolytics" helps to clear tissues like the skin from such senescent cells. In this approach it is important that the senolytic substance specifically eliminates only the senescent cells without harming the healthy dividing cells. Although senolytics remains a brand-new life science topic, the term senolytics was coined in 2015 [3], more than 700 scientific articles investigating the senolytic activity of numerous compounds have already been published. In our research collaboration between Mibelle Biochemistry and Helvecos, we have discovered an extract of alpine rose leaves (Rhododendron Ferrugineum) to exhibit senolytic activities. Based on the analysis of compounds in this extract we could identify molecules that are potentially driving this activity.

A further hallmark of aging is the depletion of stem cells [1]. Therefore, stimulating and replenishing stem cells provides another potent strategy to combat skin aging. We have previously shown that the compounds found in plant stem cells are beneficial for the vitality of skin stem cells [4]. Thus, to complement the senolytic activity of the alpine rose extract in skin rejuvenation, we studied the effect of an apple stem cell extract (Malus Domestica) in stimulating dermal progenitor cells to regenerate the dermis and the extracellular matrix. In a further experiment we also investigated the dermal-epidermal interactions induced by this apple stem cell extract. With a grape stem cell extract (Vitis Vinifera) the protection of epidermal progenitor cells could be achieved. Thus, the application of these plant stem cell extracts together with the senolytic compounds forms the powerful SenoCellTec™ concept for a deep rejuvenation of the skin.

Materials and Methods

Senolytic assay

To determine the senolytic potential of the alpine rose extract, normal human dermal fibroblasts were treated with 500 µM H_2O_2 for two hours to induce oxidative stress-induced premature senescence. After three days of culture in normal media (allowing the senescent phenotype to be fully established in a subpopulation of the cells) the mixed culture of both senescent and non-senescent cells was treated for 48 hours with either 1% alpine rose extract or Navitoclax, a known senolytic drug, or left untreated as a control. Following fixation with 2% formaldehyde and 0.2% glutaraldehyde, cell nuclei were stained with DAPI to determine the relative total cell number. A senescence-associated β-galactosidase (β-gal) activity assay was performed as described by *Zhao et al.* and a total of 400 cells were counted [5]. Treatment efficacy was determined by calculating the percentage of β-gal-positive (senescent) cells compared to the total cell number.

Chemical profiling of alpine rose leaf extract by CARAMEL

Potential senolytic molecules in the alpine rose extract were identified by chemical profiling (CARAMEL analysis, per-

formed by NatExplore SAS) [6]. The extract samples were evaporated under vacuum for 5 h (at 50 °C, 5 mbar) before being fractionated by Centrifugal Partition Chromatography (CPC, instrument TCPE300®). The obtained fractions were then analyzed by carbon-13 nuclear magnetic resonance (13C NMR, instrument Bruker Avance AVIII-600) and the metabolites contained in the extract identified. To further confirm the chemical structures of the identified metabolites the extracts were also analyzed by Liquid Chromatography Mass Spectrometry (LC-MS).

Activation of dermal Sox2 positive progenitor cells

To determine the effect of the extracts on stem cell activity, a sphere forming assay was performed. Primary dermal papilla cells were isolated from the dermis of hairy skin obtained from cosmetic surgery. After the third passage, cells were treated with 0.1% apple stem cell extract and from passage 7 sphere forming assays were performed. After 24 h, representative primary spheres were collected, and the activation of dermal progenitor cells evaluated via immunostaining for the stem cell marker Sox2. The number of secondary and tertiary spheres formed were determined after three and six weeks of cultivation, respectively.

Dermal – epidermal communication in a full thickness skin model

The effect of apple stem cell extract on the development of a rich dermal matrix was investigated using a full skin model. Primary human dermal fibroblasts were grown in submerged culture in the presence of 0.1% of the stem cell extract. After 14 days, primary human epidermal keratinocyte progenitors were seeded onto the dermal fibroblast model and grown for three days before being lifted to the air-liquid interface, followed by a further 12 days culture. The full thickness skin models were then sectioned, and dermal-epidermal organization visualized by histological analysis (hematoxylin and eosin tissue staining).

Protection of epidermal stem cells by grape stem cell extract

To evaluate the protective effects of grape stem cell extract against UV irradiation, the colony forming efficiency (CFE) was determined by means of a colony assay. Primary human keratinocyte progenitor cells were incubated with 0.625% of extract before being exposed to UV-light for 10 min at 450 kJ/m² (instrument Suntest CPS+). The number of colonies formed (parameter for active progenitor cells) was calculated and compared to unirradiated and untreated controls.

Clinical study: Effects of alpine rose leaf extract on skin redness

To test the senolytic activity of alpine rose extract *in vivo*, a randomized, placebo-controlled clinical study was performed. 44 female volunteers aged 40 to 65 years, with dull complexion and cheek redness were included in the study. After applying a cream with 2% alpine rose extract or a placebo cream twice daily on the whole face for 14 days, changes in skin redness were measured with a portable spectrophotometer (CM-700d, Konica Minolta).

Clinical Study: Effects of apple stem cell extract on skin firmness

The impact of the apple stem cell extract on skin firmness was investigated *in vivo*, in a randomized, placebo-controlled clinical study was performed with 21 volunteers (aged 35 to 56, half-side face study). The volunteers applied both a cream containing 2% apple stem cell extract or a placebo cream twice daily on a respective half side of the face. After 28 days, skin firmness was measured using an Indentometer® (IDM 800, Courage+Khazaka).

Results and Discussion

A novel senolytic active from Swiss alpine rose leaves

Fundamental for a senolytic compound is its ability to eliminate senescent cells, whilst not affecting healthy non-senescent cells. In our senolytic assay we could generate a fibroblast culture with 28% senescent cells. The treatment with the alpine rose leaf extract could significantly reduce the number of these cells to about 10%, whereas the healthy fibroblasts were mostly not affected at all **(Figure 1)**. This effect is comparable to the treatment with the known senolytic drug Navitoclax, which reduced the percentage of senescent cells to 12%. Thus, we have been able to demonstrate a very selective senolytic activity of this extract.

With our analytical analysis we aimed to establish the phytochemical profile of the alpine rose leaf extract. Indeed, we could identify numerous interesting molecules [6]. Specifically, we found taxifolin and farrerol in the extract **(Figure 2)**. These molecules are structurally very similar to fisetin **(Figure 2)**, a well-known and potent senolytic compound [7]. The natural flavonoid taxifolin causes cell cycle arrest by activating Wnt/beta-catenin signaling. This results

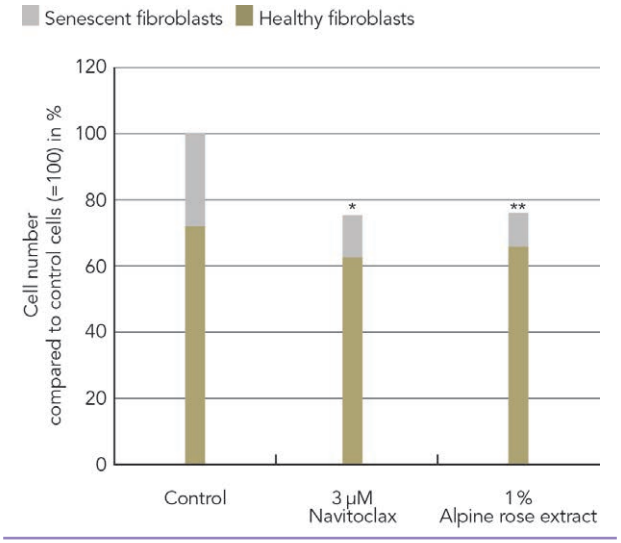

Fig. 1 Senolytic Activity of Alpine Rose Extract. Alpine rose extracted tested at 1% significantly reduced the number of senescent fibroblasts from 28.1% to 10.1%, whilst the healthy cells remained unaffected. The effect is comparable to treatment with the known senolytic drug Navitoclax.

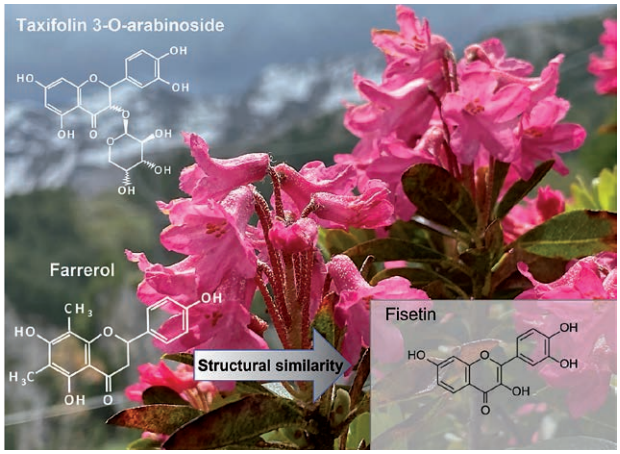

Fig. 2 Chemical Profiling and Identification of Senolytic Molecules. Chemical profiling by CARAMEL identified farrerol and taxifolin in the alpine rose extract. These molecules demonstrate a high structural similarity to fisetin, a well-known senolytic active.

in tumor regression and offers a general senolytic activity [8]. Also, farrerol exhibits senolytic functions by reducing the expression of Bcl-2 [9]. Upregulated Bcl-2 is one anti-apoptotic factor defining senescent cells' ability to be retained in the tissue, thus inhibiting Bcl-2 is a mode of senolytic action [10]. The combined presence of both taxifolin and farrerol thereby explains the senolytic activity of the Swiss alpine rose leaf extract, highlighting it as a novel senolytic derived from natural sources.

Holistic rejuvenation treatment: Activating dermal progenitor cells

The activation of dermal progenitor cells by plant stem cell extracts can support the replacement of eliminated senescent fibroblasts after a senolytic treatment. In this premise, we studied the famous apple stem cells, with proven results on the vitalization of epidermal stem cells [11], on their impact on dermal stem cell activation. We found that the apple stem cell extract was able to activate Sox2 (stem cell marker) positive dermal progenitor cells in a sphere forming assay. Further, the formation of secondary and tertiary spheres (indicative of stem-cell like activity) could be increased compared to the controls by 38% and 24% respectively (data not shown).

The treatment of dermal cells with the apple stem cell extract also had an impact on the formation of the epidermal structures and underlying dermal matrix in a full-thickness skin model. Histological sections of skin models showed that the treatment resulted in an improved columnar morphology of the basal keratinocyte layer, with an abundant underlying dermal extracellular matrix **(Figure 3a)**. Further, treatment with apple stem cell extract led to an increased dermal thickness by 27% **(Figure 3b)**.

Epidermal progenitor cells are important cells in the basal layer of the epidermis. They initiate the complete regeneration of the epidermis every three weeks and are therefore present in the upper layer of the skin. This localization however makes them also very susceptible to UV irradiation. In our studies, we found that a grape stem cell extract was able to maintain the vitality of the epidermal progenitor cells, and further stimulate their colony forming efficacy, despite UV irradiation **(Figure 4)**. In conclusion, the stem cell extracts not only have activating but also protective effects on skin progenitor cells.

Clinically proven efficacy of the natural plant extracts

To verify the observed *in vitro* results, we conducted two placebo-controlled clinical trials to study the impact of our actives on senolytic activity and skin stem cell stimulation. Senescent fibroblasts are characterized by the senescence-associated secretory phenotype (SASP) resulting in a chronic skin inflammation. In our study volunteers applying the alpine rose leave extract in a cream demonstrated a statistically significant reduction in skin redness (an indicator of skin inflammation) by 8.4% after only 14 days of treatment **(Figure 5)**. The benefits of the apple stem cell extract on the activation of dermal progenitor cells were evaluated in another clini-

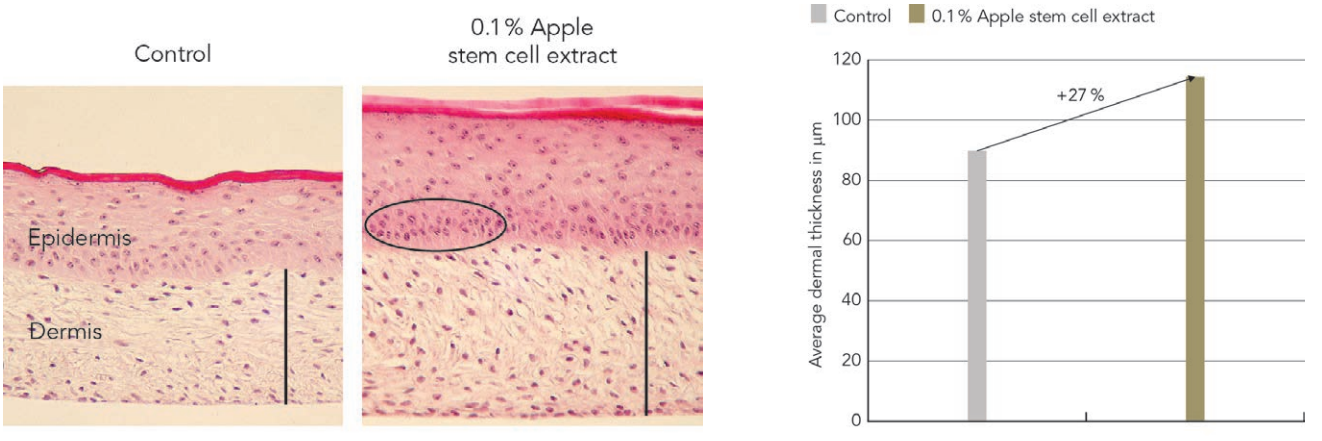

Fig. 3 Improved Dermal-Epidermal Organization with Apple Stem Cell Extract. (a) Histological sections of the full skin model analysed by H&E staining demonstrated and improved epidermis formation, with an abundant underlying dermal extracellular matrix when cells were treated with apple stem cell extract. (b) Treatment with apple stem cell extract further increased the dermal thickness by 27% compared to controls.

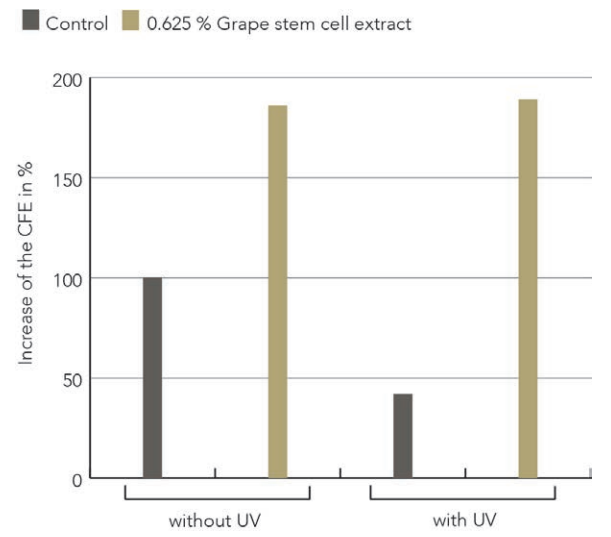

Fig. 4 Protective Effect of Grape Stem Cell Extract against UV Irradiation. Treatment with 0.625% grape stem cell extract was able to maintain the vitality of the epidermal progenitor cells and stimulate their colony forming efficacy, despite UV irradiation (450 kJ/m², 10 min).

cal study. Treatment with the extract resulted in a statistically significant improvement of the skin firmness after 28 days compared to placebo (data not shown).

Taken together, the clinical studies highlight both the potent senolytic effect of the alpine rose extract, and the stimulating effect of the stem cell extracts on skin stem cell activation and vitalization. In combination, these actives promote an improved skin structure and an overall vibrant and rejuvenated skin.

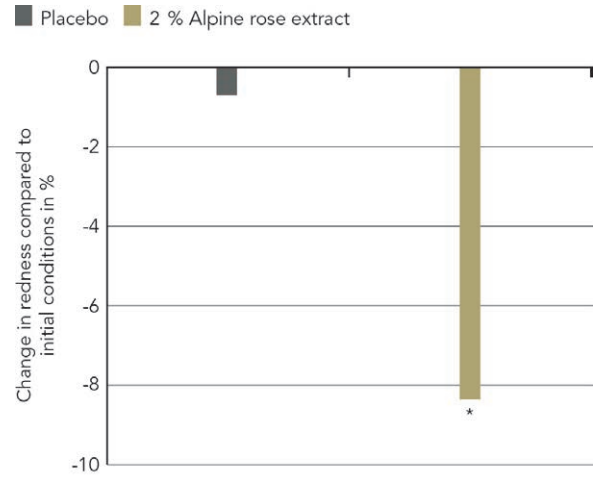

Fig. 5 Clinical Improvement of Skin Redness with Alpine Rose Extract. Application of a cream containing 2% alpine rose extract led to a significant reduction of skin redness after 14 days, compared to both placebo and initial conditions.

Conclusion

Senolytics, a new anti-aging concept, has gained a lot of attention in the last few years. The specific elimination of senescent fibroblasts in the skin without harming healthy cells is also a very attractive strategy for cosmetics. In our studies, we have shown that an extract of alpine rose leaves exerts an excellent senolytic activity, comparable to established senolytic drugs. To further develop this anti-aging strategy, we have developed SenoCellTec™, an active ingredient concept built on the idea of removing senescent cells from the skin, and then in turn vitalizing and protecting the skin stem cells to boost the rejuvenation process. By combining the use of the senolytic alpine rose extract and the activating and vitalizing stem cell extracts, this "clear and fill" concept provides a new and revolutionary concept for holistic skin rejuvenation, with promising treatment options in the very near future.

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