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Understanding Solar Skin Elastosis–Cause and Treatment

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Synopsis

Photoageing, also called actinic ageing, is the main cause of prematurely aged skin. Our expertise in elastic fibers has led us to discover a process triggered in response to ultraviolet (UV) light and which upsets the balance of elastin fibers: there is too much elastin and insufficient lysyl oxidase (LOXL1) enzyme to form functional elastic fibers. This imbalance then leads to an accumulation of nonfunctional elastin, which forms aggregates. In addition to this imbalance, UV rays also induce elafin synthesis by fibroblasts. Known to be a marker of elastotic aggregates, elafin crystallizes the elastin fibers and stimulates the formation of aggregates that cannot be naturally eliminated by the skin. We developed a *Hamamelis virginiana* leaf extract that was able to restore both the balance between elastin and LOXL1 and to decrease the elafin synthesis to fight and correct the damage. This specific *Hamamelis virginiana* extract increased LOXL1 expression by twofold and decreased elafin synthesis. As a consequence, elastic fibers became functional and aggregates of unfunctional fibers decreased. The specific Hamamelis extract activity was confirmed *in vivo* with decreasing wrinkles and improving skin firmness.

INTRODUCTION

Cutaneous ageing involves two independent biological processes: chronological (intrinsic) ageing and extrinsic ageing, the latter of which accelerates normal intrinsic ageing. Tobacco, environmental factors (cold temperature, pollution, and UV rays), mechanical factors, and nutritional factors cause extrinsic ageing (1). However, photoageing (induced by UV radiation) is the main cause of prematurely aged skin, also called actinic ageing. Photoageing principally concerns the exposed parts of the body. The visible effects of actinic ageing depend on the type of individual and are determined by the day-to-day exposure and protection from the sun. During actinic ageing, the epidermis is characterized by an irregular thickness, either hyperplasic or atrophic (2). As the stratum corneum thickens (keratosic aspect), the superficial layers dehydrate. When melanocytes are exposed to the sun, their numbers decrease and they are not uniformly spread over the skin, resulting in irregular pigmentary spots. As a consequence, basal cells lose their protection leading to a possible cutaneous carcinoma. Moreover, the dermis is also largely affected by UV radiation. UV-induced ageing is characterized by a significant alteration of conjunctive tissue at the collagen and elastic fiber levels (3).

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Solar elastosis is considered to be a sign of chronic sun exposure–related ageing, which is histologically characterized by reduced numbers of dermal collagen fibers and the accumulation of dystrophic elastotic material (4). The elastin fibers attach themselves forming elastotic aggregates, which form abnormal nonfunctional disintegrated elastic fibers, leading to a premature ageing phenotype, associated with loss of elasticity, yellow skin, and deeper wrinkles. With ageing, elastic fibers are abundant, thickened, and disorganized.

Elastic fibers are formed mainly during embryonic development with the deposition of tropoelastin, the soluble precursor of elastin, on a scaffold of fibrillin rich in microfibrils (5–7). The lysyl oxidase (LOXL1) catalyzes the formation of covalent cross-links between some lysine residues of two adjacent tropoelastin molecules, which become insoluble polymer elastin. Mature elastic fibers consist of an external coat of microfibrils and an amorphous core of cross-linked elastin. These cross-linked amino acids consolidate the polymer, procure its elastic function, and ensure its resistance. The cross-linking step is essential to strengthen the growing elastic fibers, and this highlights the important role played by LOXL1 in elastogenesis.

Moreover, elafin, also called skin-derived antileukoproteinase, is a serine protease inhibitor, mostly produced by epithelial cells. In the skin, keratinocytes are the main source of this molecule. Although elafin is not detectable in normal skin, it is secreted abundantly in psoriasis and other inflammatory skin disorders (8). Elafin acts in various ways on the cutaneous immune homeostasis by not only exerting antiprotease but also by causing immunomodulatory and antiproliferative effects. In the actinic elastosis of sun-damaged skin, fibroblasts express elafin, (also known as peptidase inhibitor), which binds to elastin. The elafin–elastin complex hampers the elastolytic regular process, leading to the accumulation of abnormal disintegrated elastic fibers and aggregates (9).

The accumulation of elastotic material can be caused by multiple factors, such as a decrease in collagen, an increase in the synthesis of elastin and/or a breakdown of existing and nascent elastic fibers.

We proposed in this study to rebalance the key partners involved in the solar elastosis mechanism to improve the level of functional elastic fibers in the skin exposed to sun radiation.

We tested the effect of our new specific Hamamelis extract on LOXL1 expression. By increasing LOXL1 expression, our specific Hamamelis extract balances elastin/LOXL1 expressions for getting more functional elastin fibers.

The potential of our specific Hamamelis extract to counteract the accumulation of abnormal elastin fibers was also evaluated by measuring its capacity to inhibit elafin expression.

Thanks to this clinical study, we have shown that our specific Hamamelis extract maintains youthful skin when exposed to the sun and may be used as a treatment applied at night to relieve the harmful effect of the sun light during the day.

METHODS

IN VITRO BIOLOGICAL TESTS

Active ingredient. In vitro: Hamamelis extract is an extract of leaves of *Hamamelis virginiana*.

Clinical trial. Hamamelis is a formulation containing 1% of water, pentylene glycol, *Hamamelis virginiana* (witch hazel) leaf extract, xanthan gum, and caprylyl glycol.

REAL-TIME PCR FOR ANALYSIS OF ELASTIN AND LOXL1 EXPRESSION LEVELS

Cell culture. Fibroblasts (63-year-old donor) were cultured until confluence. They were then exposed to ultraviolet A (UVA) (7.5 J/cm^2) and cultured for 24 h. LOXL1 and elastin expression was evaluated by quantitative - reverse transcription - polymerase chain reaction (q-RT-PCR).

In the second experiment, fibroblasts (63-year-old donor) were cultured until confluence. Three different conditions have been used. The control culture was maintained for 24 h and then exposed to UVA (7.5 J/cm^2). The culture was maintained for 16 h. In the post irradiation condition, the Hamamelis extract was added just after the irradiation, and maintained for 16 h. In the pre- and postirradiation conditions, the specific Hamamelis extract was added in the culture at confluence and for 24 h. Then the culture was exposed to UVA (7.5 J/cm^2) and the Hamamelis extract was added for additional 16 h.

Assay method. The cells were washed with phosphate buffer solution (PBS) and then total RNA was extracted (Spin Vacuum Total RNA Isolation System Z3500, Promega, Charbonniere les bains, France). Total RNA content and quality were evaluated by measuring optical density at 260 and 280 nm. Real-time RT-PCR was performed using iScript one-Step Real-time reverse transcription-polymerase chain reaction (qRT-PCR) Kit with SYBRgreen (Bio-Rad, Marnes-la-Coquette, France). The following primers were used for PCR:

For LOXL1.

Forward 5′-GACTTCGGCAACCTCAAGC-3′

Reverse 5′-TGTTGCAGAAACGTAGCGAC-3′

For elastin.

Forward 5′-GTGTATACCCAGGTGGCGTG-3′

Reverse 5′-CGAACTTTGCTGCTGCTTTAG-3′

All primers were in separate zone of the exon. Amplification was performed with 40 cycles, measuring the fluorescence at the end of each cycle. The comparative Ct method (f¢Ct) was used for relative comparison. Real-time PCR experiments were calibrated with actin as the housekeeping gene. As negative controls, samples without RNA were used in the same conditions. Results were expressed related to control (untreated irradiated cells) and normalized to actin.

Results and statistics. The results are expressed as percentage compared with untreated control and then expressed as mean ± standard deviation (SD) from nine replicates. The statistical analysis was carried out using a Student *t*-test.

IMMUNOHISTOCHEMISTRY: ELAFIN IN NORMAL HUMAN BIOPSIES EXPOSED TO UVA

Cell culture. Human biopsies (abdominal part) from a 27-year-old donor were cultured in a specific defined medium at 37°C, with 5% CO_2, for 10 d. The biopsies were either irradiated or not with UVA at 5 J/cm^2 during a period of 10 d. UVA were applied from day 2 with and without our specific Hamamelis extract at 0.5%, every day in a topical or systemic manner.

Assay method. Samples were fixed in a formalin solution and they were then dehydrated and embedded in paraffin. Seven-micrometer sections were deparaffinized.

The antihuman elafin antibody (Abcam) was used at a concentration of 1/200 for one night at room temperature and amplified with streptavidin/biotin. The technique involves three layers. The first layer is an unlabeled primary antibody. The second layer is a biotinylated secondary antibody. The third layer is a complex of streptavidin–biotin peroxidase. The peroxidase (CliniSciences, Nanterre, France) produces purple-colored end products. They were observed using an optical microscope (Leica DMLB, Nanterre, France).

Quantification. The staining was detected with acquisition of a threshold that allowed selecting the staining in the interest area. The surfaces were then measured. The data were exported as values in an Excel file.

Results and statistics. The quantification of each band was relative to actin. The results were expressed in the protein percentage compared with the untreated control at 100%.

Statistics were analyzed using a Mann–Whitney nonparametric test.

IN VIVO CLINICAL TESTS

Study design. The clinical study was carried out as a placebo-controlled double-blind randomized split-face study under dermatological control. The efficacy of the formula containing Hamamelis at 1% was compared with the baseline (before treatment, D0) and to the other half-face treated with placebo. The study was conducted during a period of 56 d with check points at D0, D28, and D56 for the antiwrinkle effect, and during a period of 84 d with check points at D0 and D84 for the firming effect. This study was carried out in France from March to June 2013.

Inclusion criteria. The study was performed on Caucasian female volunteers aged from 52 to 76 year, displaying wrinkles and fine lines on the crow's feet of average to strong intensity and saggy skin on the face.

Application modality. The products (containing Hamamelis at 1% or placebo formula) were applied by the volunteers twice a day, on each half face for 84 d. The application was carried out by the volunteers, by circular massages until complete penetration, especially on the crow's feet area and the cheek.

METHOD OF EVALUATION

Measurement of skin roughness by fringe projection. Images were acquired on the crow's feet area (surface of 12 cm^2) via digital video camera coupled to a fringe projection system (Dermatop™ system, Breuckam, Meersburg, Germany, Eotech, Marcoussis, France) at D0, D28, and D56. ST and Stm parameters were calculated as follows (TopoSurf and Optocat analysis systems):

- ST: maximum amplitude of the relief (mm). A decrease means a reduction of the main wrinkles.
- Stm: mean difference between peaks and valleys (mm). A decrease means a smoothing of the studied surface.

Measurement of the firmness of the skin with Dynaskin®. Dynaskin® (Orion Concept, Tours, France) is an add-on to the DermaTop™ system, which permits to evaluate the

firmness of the skin with a noncontact method. Dynaskin® produces a deformation close to the clinical approach by blowing air perpendicular to the area of interest or with a dedicated angle of 45°. The 3D sensor, using fringe projection techniques, captures just before the deformation of shape of the local surface and then when the deformation is applied. The parameters studied are the volume (mm^3) and the maximal depth (mm) of the deformation. A decrease in these parameters shows an improvement in the firmness of the skin.

Results and statistics. The results were expressed with the mean percentage of variation of the mean value of measured parameters: (meanDx – meanD0)/meanD0. The mean percentages of variation compared with placebo were also calculated as mean variation of Hamamelis at 1% mean variation placebo.

The normality of distribution was checked using Shapiro–Wilk test (level of significance at 1%). The statistical comparisons of the evolution of the parameter with time and with the placebo have been performed with Student *t*-test (if the normality of distribution is confirmed) or with the nonparametric Wilcoxon test or Mann–Whitney test (if the normality of distribution is rejected). The level of significance is 5%.

RESULTS

TOO MUCH ELASTIN AND LACK OF LOXL1 UNDER UVA IRRADIATION IN THE DERMIS: AN INEFFICIENT BALANCE

Using quantitative RT-PCR, we have shown in fibroblasts that under UVA radiation, the expression of elastin mRNA is increased by 7.5-fold, whereas the one of LOXL1 mRNA remains stable (Figure 1). This imbalance between the expression of elastin and LOXL1 suggests that elastin is not conveniently cross-linked and therefore that the amount of functional fibers synthesized under UVA radiation is insufficient.

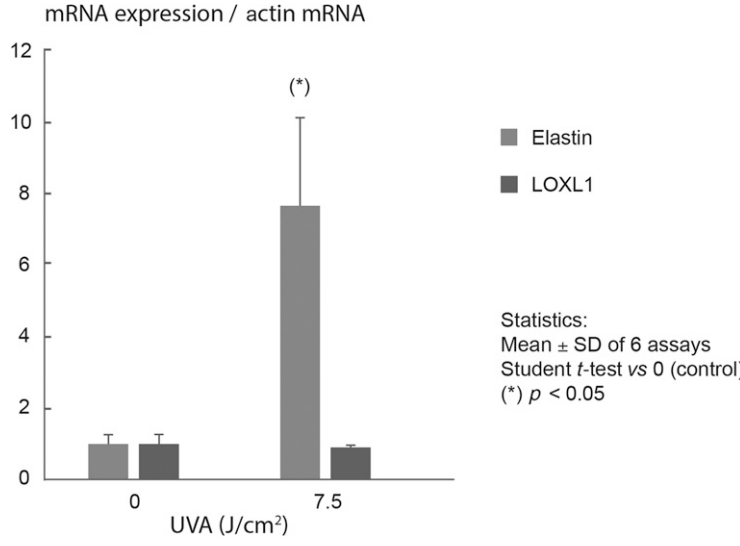

Figure 1. Expression of LOXL1 and elastin mRNA in UVA-irradiated fibroblasts versus nonirradiated cells (0).

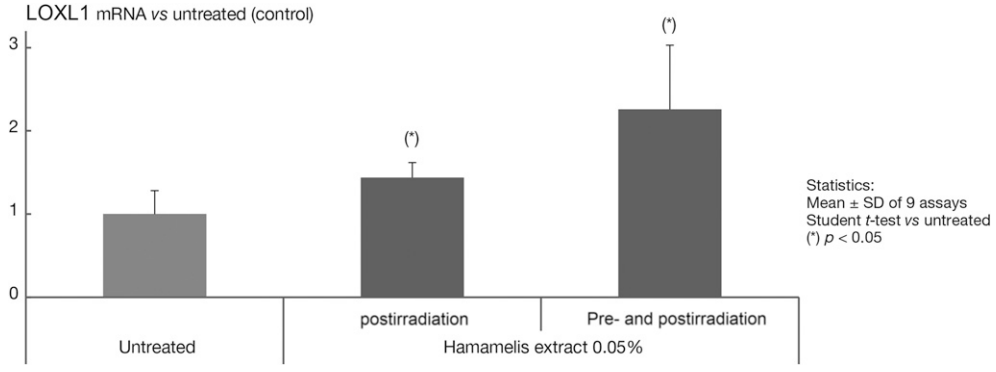

Figure 2. Induction of LOXL1 mRNA with a specific Hamamelis extract at 0.05% pre- and postirradiation with UVA exposure at 7.5 J/cm^2 versus irradiated control (not treated with plant extract).

OUR SPECIFIC HAMAMELIS EXTRACT BALANCES ELASTIN—LOXL1 EXPRESSION TO OBTAIN FUNCTIONAL ELASTIN FIBERS

Results showed that our specific Hamamelis extract at 0.05% induces LOXL1 mRNA expression by twofold in UVA-treated fibroblasts when the specific Hamamelis extract was added just after UVA exposure/treatment and when the specific Hamamelis extract was added 24 h before UVA and just after UVA (pre- and postirradiation).

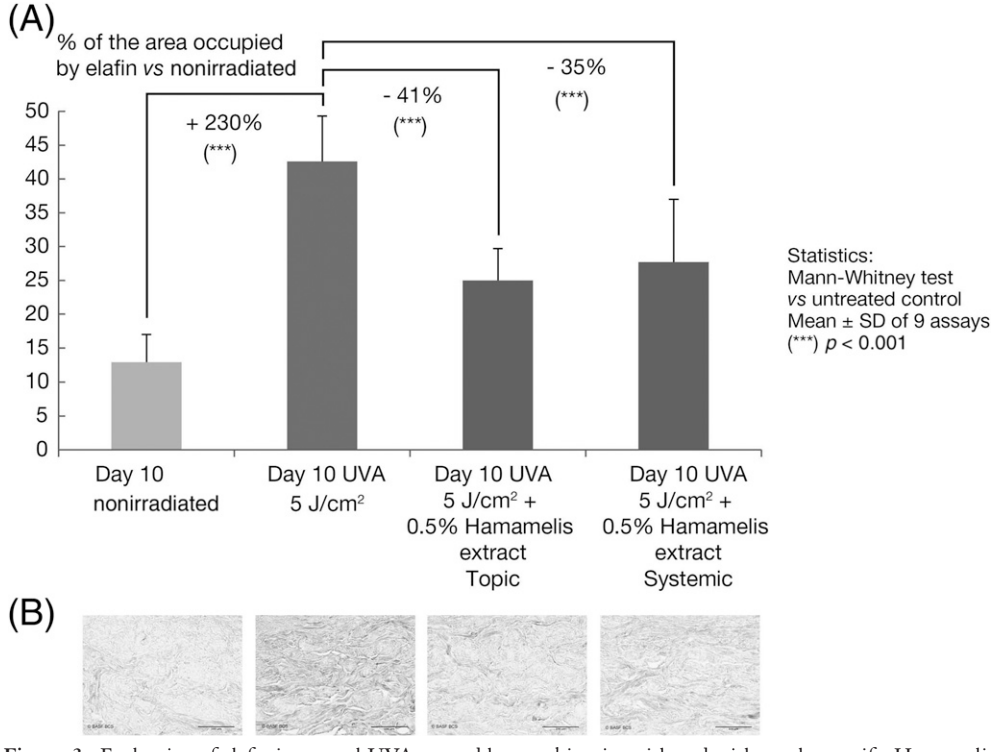

Figure 3. Evaluation of elafin in normal UVA-treated human biopsies with and without the specific Hamamelis extract. (A) Quantification of elafin staining in purple in the dermis. (B) Corresponding images.

LOXL1 mRNA was significantly increased with the Hamamelis and UVA-treated cell culture compared with the untreated control (Figure 2).

THE SPECIFIC HAMAMELIS EXTRACT DECREASES THE SYNTHESIS OF ELAFIN, RESULTING IN THE ELIMINATION OF ELASTOTIC AGGREGATES

Results showed that UVA exposure significantly induced by 230% the expression of elafin in UVA-treated normal human biopsies. The specific Hamamelis extract at 0.5% inhibited elafin expression in normal UVA-treated human skin sample, regardless of the manner Hamamelis extract is added (topically or systemically).

Results were significant when compared with the UVA-treated biopsies: topically—41% and systemically—35% (Figure 3).

Each bar of the histogram represents the quantification of the elafin purple staining in the corresponding image in the dermis. We can see greater staining in UVA-treated skin samples compared with the nonirradiated and less of elafin staining in the UVA-treated skin samples treated with the specific Hamamelis extract compared with the staining of UVA-treated skin samples without the specific Hamamelis extract.

CLINICAL TESTS

In a placebo-controlled clinical study, we evaluated by fringe projection the ability of Hamamelis to reduce wrinkles. Using Dynaskin®, we checked if Hamamelis extract can improve the firmness of the skin on female volunteers presenting wrinkles and/or fine lines on the temples or crow's feet area and a sagging facial skin.

HAMAMELIS: ANTIWRINKLE EFFECT AGAINST CROW'S FEET AREA (BY FRINGE PROJECTION)

After applying the emulsion containing Hamamelis at 1% on one half of the face, we observed that the depth of the main wrinkles was reduced (ST) and the mean roughness

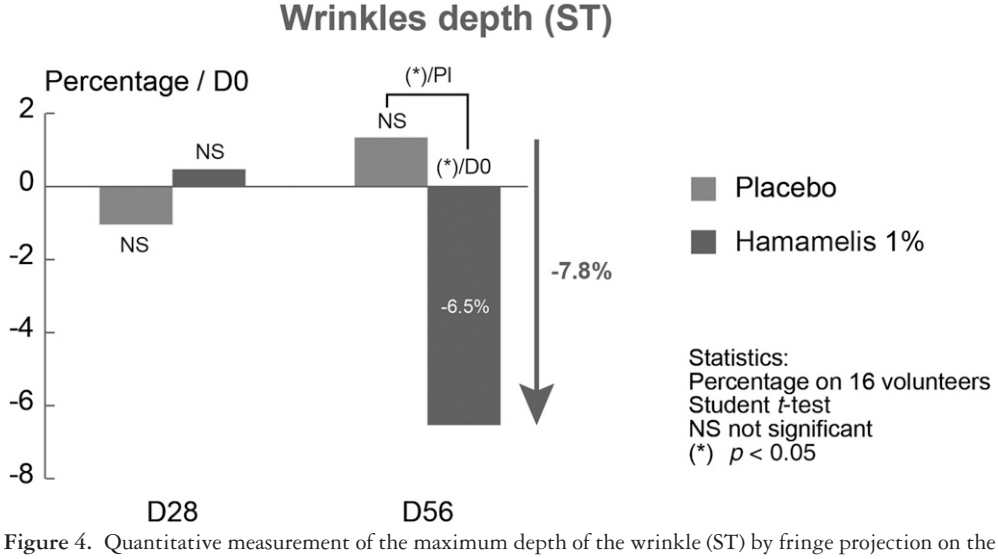

Figure 4. Quantitative measurement of the maximum depth of the wrinkle (ST) by fringe projection on the crow's feet. Emulsion with Hamamelis extract at 1% and placebo. Mean percentage of change of ST compared with the baseline (D0).

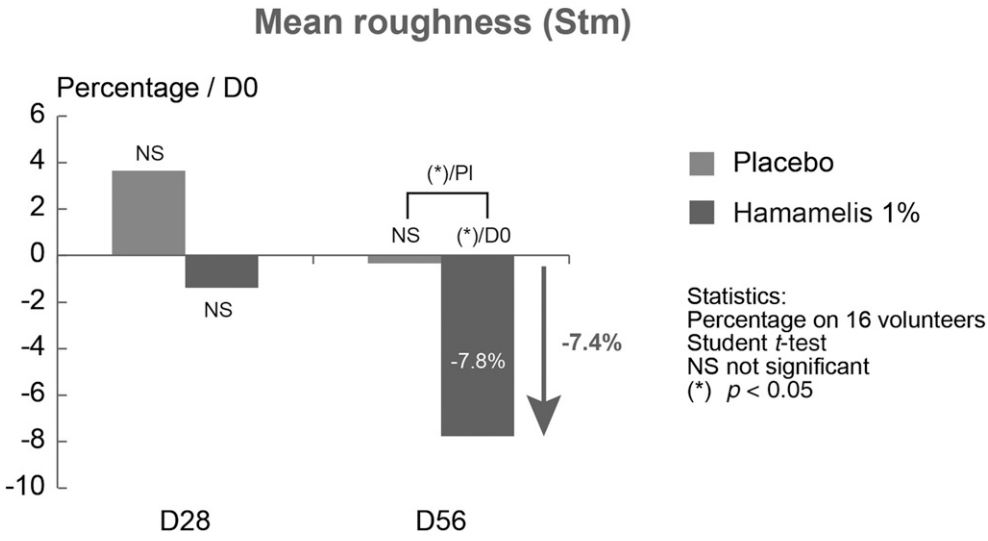

Figure 5. Quantitative measurement of the mean difference between peaks and valleys (Stm) by fringe projection on crow's feet wrinkles. Emulsion with Hamamelis at 1% and Placebo emulsion. Mean percentage of evolution of Stm compared with the baseline (D0). Statistics versus D0 and placebo (Pl).

(Stm) in the crow's feet areas. This observation is statistically significant compared with the baseline (D0) and the placebo at D56. After 56 d of applying the emulsion, the depth of the main wrinkles was reduced by 6.5% compared with the baseline and by 7.8% compared with the placebo ($p < 0.05$) (Figure 4). Moreover, the mean roughness was reduced by 7.8% compared with the baseline (D0) and by 7.4% versus the placebo ($p < 0.05$) (Figure 5).

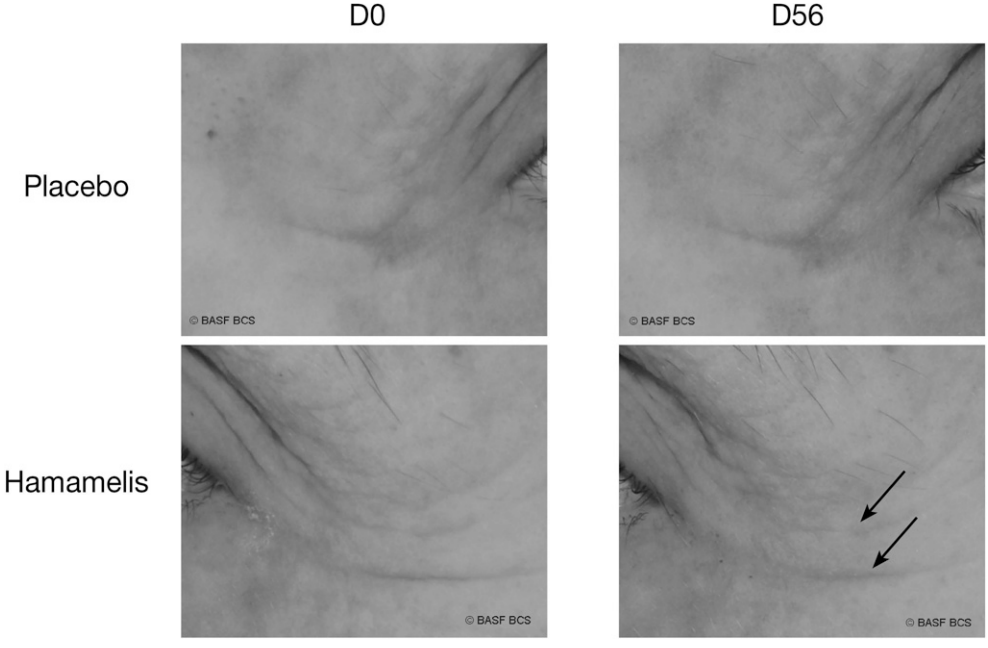

Figure 6. Photographs of a 56-year-old donor.

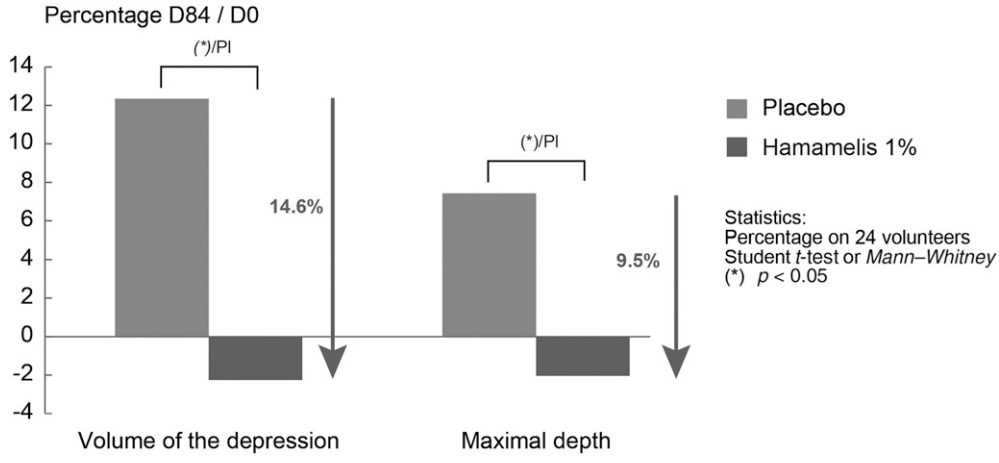

Figure 7. Quantitative measurement of the volume of the depression and the maximal depth by Dynaskin® on the cheek. Emulsion with Hamamelis at 1% and emulsion with placebo. Mean percentage of evolution of volume of the depression and the maximal depth compared with the baseline (D0). Statistics versus placebo (Pl).

This antiwrinkle effect after 56 d of application of the emulsion containing Hamamelis at 1% is illustrated in the photographs in Figure 6.

HAMAMELIS FIRMS THE SKIN OF THE FACE (DYNASKIN®)

The depression created by airflow on the cheeks was measured to evaluate the firming effect of the emulsion containing Hamamelis at 1%. The volume and the depth parameters of the deformation were measured.

Compared with the placebo, after 84 d of treatment with Hamamelis at 1%, we observed a significant reduction of the volume of the depression by 14.6% ($p < 0.05$) and a significant reduction of the maximal depth of the deformation by 9.5% ($p < 0.05$) (Figure 7). This lower deformation of the skin created by the airflow, compared with the placebo, indicates a firming effect of Hamamelis at 1%.

DISCUSSION

Year after year, the slackness of the skin becomes more and more noticeable. The lack of elasticity of the skin is one of the major signs of ageing, whether it is caused by time or accelerated by exposure to the sun rays. With age and sun, the oxidative phenomenon leads to the densification of the collagen fiber network and to the degradation of elastin fibers. The dermis gets thinner and the elastic fibers split, which lead to a lack of skin elasticity. Wrinkles and fine lines appear.

UV radiations increase the synthesis of metalloproteases, which degrade many components of the extracellular matrix, including collagen IV and VII and functional elastic fibers. These phenomena involve leveling of the dermoepidermal junction and a significant alteration of the conjunctive tissue. Elastin fibers are in large numbers, thickened, and tangled. The fibers stick together to form aggregates that are present throughout the dermis. We called this phenomenon as solar elastosis. In these conditions, elastin is badly

reticulated by LOXL1. Indeed, we have shown that LOXL1 is downregulated under UV radiation. Moreover, LOXL1 has been shown to be degraded by skin elastases. Abnormal and nonfunctional fibrils appear.

Furthermore, elafin is a serine protease inhibitor that is mostly produced by epithelial cells. In the skin, keratinocytes are the main source of this molecule. Although elafin is not detectable in normal skin, it is abundantly secreted in psoriasis and other inflammatory skin disorders (10). Elafin acts in a varied way on the cutaneous immune homeostasis. Elafin does not only exert an antiprotease effect but also an immunomodulatory and antiproliferative one (11). In solar elastosis, fibroblasts also express elafin, which binds to elastin. The elafin–elastin complex limits the elastolytic regular process, leading to the accumulation of disintegrated abnormal elastic fibers and aggregates. The UV-induced elafin prevents the proteases to degrade the abnormal fibers (9). By inhibiting the synthesis of elafin, our specific Hamamelis extract gives access back to proteases, to degrade the abnormal fibers.

Therefore, the specific Hamamelis extract acts at two levels: (i) it makes more functional elastin fibers by increasing the elastin–cross-linked enzyme LOXL1 and (ii) prevents the formation of the nonfunctional elastin fibers by inhibiting elafin, the peptidase inhibitor. The *in vitro* results are confirmed with the clinical data which show a decrease in wrinkles and an improvement in the firmness of the skin.

CONCLUSION

Complementarily to UV filters' sun protection, we developed an active ingredient able to give the skin its own power to fight against the damage caused by the sun and to repair the already existing ones. Thanks to our knowledge on elastic fibers, we discovered a process triggered in response to UV light, which upsets the balance of these fibers: too much elastin and too little LOXL1 enzyme to assemble them into functional elastic fibers. This imbalance leads to the accumulation of nonfunctional elastin, which groups together in aggregates and cannot be naturally removed. In addition to this imbalance, the fibroblast synthesizes elafin, known to be a marker of elastotic aggregates. This protein crystallizes the elastin fibers and emphasizes the formation of aggregates, which cannot be naturally eliminated by the skin.

We have designed the first active ingredient based on *Hamamelis virginiana* leaf extract (witch hazel) that corrects the damage caused by solar elastosis and that acts as a powerful shield against photoageing by acting on these two phenomena: the imbalance between LOXL1 and elastin, and the overexpression of elafin.

The active ingredient efficacy is proven: lines are decreased and the skin recovers its firmness.

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