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A new decorin-like tetrapeptide for optimal organization of collagen fibres

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Synopsis

Decorin interacts with collagen via its protein core and influences collagen fibrillogenesis, thus regulating excessive bundle-like aggregation of collagen. As skin ages, there is lack of functional decorin, which results in disrupted collagen fibres and in a reduction in the tensile strength of the skin. Therefore, a substitute for decorin would make up for the non-functional decorin that is present as we age. Two tetrapeptide sequences have been identified as the specific binding sites of decorin to collagen fibrils. These sequences were engineered to generate new tetrapeptides with improved affinity that would present a decorin-like activity. A focused library of several candidates was synthesized containing only tetrapeptides that mimicked the binding sequences of decorin. The candidates were screened with an *in vitro* collagen fibrillogenesis assay and the tetrapeptide with International Nomenclature of Cosmetic Ingredients (INCI) name *Tripeptide-10 Citrulline* achieved the best results. Like decorin, this synthetic tetrapeptide proved, through *in vitro* tests, to regulate collagen fibrillogenesis and to influence the diameter of collagen fibres, making them thinner and more uniform. *Tripeptide-10 Citrulline* is a new cosmetic active to target specifically collagen fibre organization. Skin collagen quality is addressed rather than skin collagen quantity. *Tripeptide-10 Citrulline* ensures uniformity in fibril diameter and increases skin suppleness because of a better cohesion of collagen fibres.

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In line with the Operations Manual of the IFSCC, this 2007 IFSCC Conference Award winning paper will also be published in the IFSCC Magazine.

Résumé

La décorine réagit avec le collagène par sa partie protéinique et influence la fibrillogénèse du collagène, régulant ainsi l'agrégation en paquets excessive de ce dernier. Lorsque la peau vieillit, il se produit une perte en décorine fonctionnelle, ce qui provoque une désorganisation des fibres du collagène et une diminution de la force en traction de la peau. Ainsi, un remplaçant de la décorine compenserait la décorine non fonctionnelle qui apparait lorsque nous vieillissons. Deux séquences tétrapeptidiques ont été identifiées comme étant les sites spécifiques de liaison de la décorine aux fibrilles de collagène. Ces séquences ont été reproduites pour générer de nouveaux tétrapeptides avec une affinité améliorée, ce qui conduit à une activité décorine like. Une librairie de plusieurs candidats a été synthétisée, contenant uniquement les tétrapeptides qui simulent les séquences de liaison de la décorine. Les candidats ont été « screenés » au travers d'un test in vitro de fibrillogénèse du collagène et le tétrapeptide présentant le nom INCI *Tripeptide-10 Citruline* a conduit aux meilleurs résultats. Comme la décorine, ce tétrapeptide synthétique s'est montré capable, au travers de tests in vitro, de réguler la fibrillogénèse du collagène et d'influencer le diamètre des ses fibres, les rendant plus fines et plus uniformes.

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Le *Tripeptide-10-Citruline* est un nouvel actif cosmétique pour cibler spécifiquement l'organisation des fibres de collagène. Il s'adresse plutôt à la qualité du collagène de la peau plutôt qu'à sa quantité. Le *Tripeptide-10 Citruline* assure une uniformité dans le diamètre des fibrilles et augmente la souplesse de la peau en améliorant la cohésion des fibres de collagène.

Introduction

Collagen fibrils in skin are composed primarily of type I collagen with minor amounts of type III and V collagen. In fibrillar collagens (such as type I collagen), the polypeptide chains, called α-chains, are synthesized in the shape of procollagen, a triple helix with long loose ends. Procollagen is secreted into the extracellular space where most of the non-helical ends are enzymatically removed. This allows the shortened molecules, now called tropocollagen, to assemble into ordered polymers called collagen fibrils, which are thin structures (<300 nm in diameter), many hundreds of micrometers long in mature tissues and clearly visible in electron micrographs. This process is called fibrillogenesis. The collagen fibrils often aggregate into larger, cable-like bundles, which can be seen in the electron microscope as collagen fibres several micrometers in diameter.

Fibrillogenesis is an essential process in tissue formation, but must be controlled and regulated to avoid excessive bundle-like aggregation of collagen. One of the main molecules responsible for fibrillogenesis control is a proteoglycan called *decorin.*

Decorin belongs to a growing family of structurally related proteoglycans, grouped as the small leucine-rich proteoglycans that are directly involved in the control of matrix organization and cell growth.

Decorin works by inserting itself between two parallel neighbouring collagen molecules in the fibril, helping to stabilize them and to orient fibrillogenesis. As decorin binds to the surface of collagen fibrils, it delays the lateral assembly of individual triple helical collagen molecules and the diameter of the fibrils is decreased [1]. This controls fibril dimensions, uniformity of their diameter and their regular spacing, thus helping to establish and maintain tissue shape.

The binding of decorin to one collagen triple helix is proposed to play a major role in the formation of the staggered arrangement of collagen molecules within the microfibrils by preventing lateral fusion of collagen molecules (Fig. 2). Decorin governs collagen fibril growth and influences higher order matrix assembly.

Structurally, decorin is similar to a horseshoe where the inner concave surface is formed by β-sheets and the outer convex face is made up of α-helices. The inner concave surface of decorin is of suitable size to accommodate a single triple helix of collagen.

Decorin is associated with collagen fibrils at specific binding sites in the protein core. There are two collagen binding sites on decorin, one on each arm of the horseshoe (see Fig. 1). Two tetrapeptide sequences (HLEK and HLER) have been identified in the literature as the specific binding sites of decorin to collagen fibrils [2].

The importance of decorin modulation in collagen fibrillogenesis has been shown in decorin null mice: homozygous animals have fragile skin. Decorin-deficient mice are characterized by skin with a reduced tensile strength, containing collagen fibrils with irregular profiles caused by lateral fusion and a thinner than normal dermis [3].

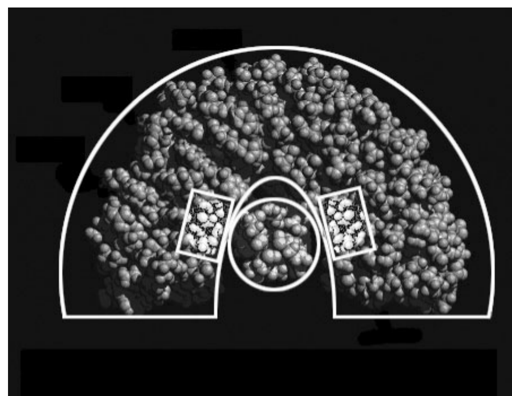

Figure 1 Model of decorin (horseshoe) complexed with a triple helix of collagen (circle). Binding sites are shown (rectangles).

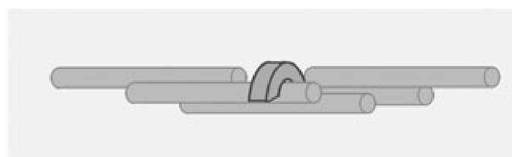

Figure 2 Decorin acts as a spacer and prevents lateral fibril fusion.

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Collagen orientation is also affected: in the absence of decorin, fibrils in the ligament display a random orientation instead of their usual parallel orientation [4].

These studies conclude that decorin is essential in the skin to assure its functional morphology and physical characteristics by controlling collagen aggregation, homogenizing fibril diameters and maintaining a regular packing (Fig. 5).

However, the ageing process induces significant changes in the proteoglycans present in the skin. The most pronounced change in skin proteoglycans is the appearance in mature skin of a catabolic fragment of decorin named decorunt [5]. This truncated form of decorin, not found in young skin, lacks regions previously shown to be important for interaction with collagen. The appearance of this catabolic product may have a significant effect on skin elasticity and morphologic differences between collagen fibres of young and mature skin [6].

In this investigation, twenty tetrapeptides that mimicked the collagen-decorin binding sequences were synthesized. Candidates were selected and tested with the aim to find a peptide with decorin-like functionality that could be used as a substitute for the non-functional decorin present in aged skin.

Materials and methods

Tetrapeptide synthesis

Twenty-three tetrapeptides were synthesized by solid phase synthesis, most of which replicated the following charge pattern: +, −, 0, +. This pattern is identified in the literature as being necessary for decorin–collagen binding [2]. The tetrapeptides were screened *in vitro* as fibrillogenesis modulators in the following assay.

Collagen fibrillogenesis modulation

Kinetics assay

Type I collagen from calf skin (Sigma, St. Louis, MO, USA) was dissolved in 17 mmol L^{-1} acetic acid at the concentration of 2 mg mL^{-1}. Thirty microlitres of this solution was added to a 96-well

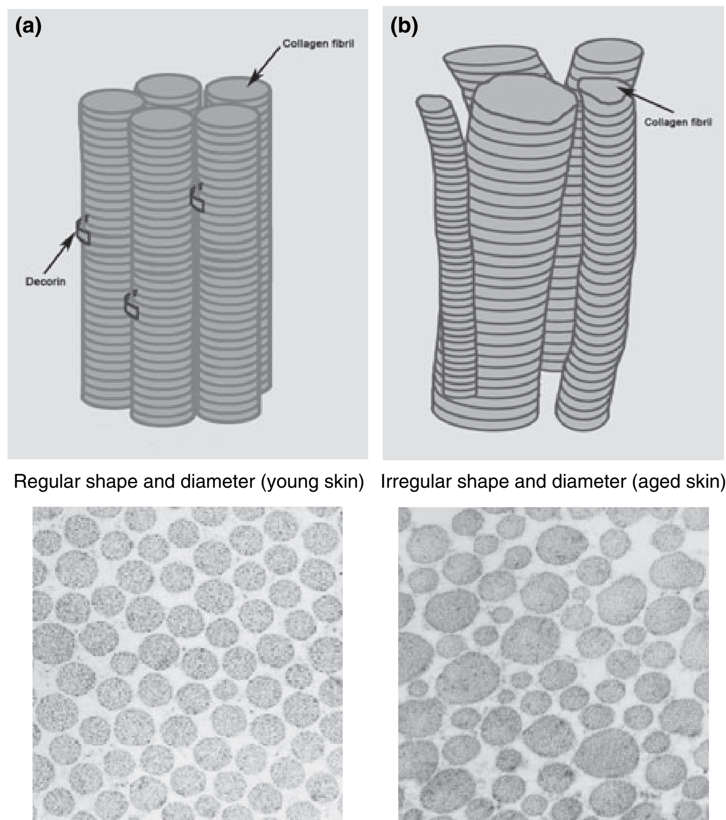

Figure 3 Fibril assembly with decorin (a) and without decorin (b) as shown in a diagram (top) and in microscopy images, from [3] (bottom).

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plate, with 20 μL per well of the peptide solution (10 mg mL^{-1}), and the volume was completed with 150 μL per well of fibrillogenesis buffer (0.12 mol L^{-1} NaCl and 30 mmol L^{-1} sodium phosphate, pH = 7.3). The final concentration of the tested peptides in this assay was 0.1% (w/v). The plate was incubated at 30°C and the process of fibrillogenesis was measured as a function of turbidity by monitoring the change in absorbance at 405 nm at 30-min or 1-h intervals. The values obtained were compared with a negative control lacking the test peptide.

Dose–response assay

This assay was performed as described above, at different peptide concentrations. Final concentrations of the target peptides in the samples were 0.001%, 0.01%, 0.05% and 0.1% (w/v). One candidate was selected for further testing.

Study of collagen fibril diameter in a skin model

Organotypic cultures

Human skin tissue models were supplied by Mattek Corporation (Ashland, MA, U.S.A.). EpidermFT full thickness skin model consists of normal, human-derived epidermal keratinocytes and normal, human-derived dermal fibroblasts, which have been cultured to form a multilayered, highly differentiated model of the human dermis and epidermis. Upon reception, tissues were returned to culture at 37°C, 5% CO_2, after dispensing 2 mL per well of EFT-200 medium. Cultures were allowed to equilibrate overnight before the first product application.

Product application

After the equilibration period, the organotypic cultures were incubated for 14 days in the absence (negative controls) or in the presence of test product. The culture medium was changed every other day. *Tripeptide-10 Citrulline* was diluted in EFT-200 medium at a final concentration of 0.01% (w/v) and applied every 48 h within 14 days (on days 1, 3, 5, 7, 9, 11 and 13).

Transmission electron microscopy (TEM)

On day 14, organotypic cultures were fixed in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer, pH 7.4. The tissues were then post-fixed in 1% osmium tetraoxide. Following fixation, samples were dehydrated in graded ethanol and infiltrated with Spurr's resin. After polymerization of the resin, thick sections were produced using a Reichert Ultracut E microtome (LEICA Microsystems GmbH, Wetzlar, Germany) and sections were stained with methylene blue to determine orientation. The blocks were then thin sectioned and mounted on gold grids. Grids were stained with 2% uranyl acetate in deionized water and Reynold's lead citrate. Stained grids were examined at various magnifications using a Jeol JEM 1010 TEM (Jeol, Tokyo, Japan). The diameter of collagen fibres of two areas randomly chosen from each sample was measured. The data obtained from the measurements were statistically analysed using a one-way ANOVA method.

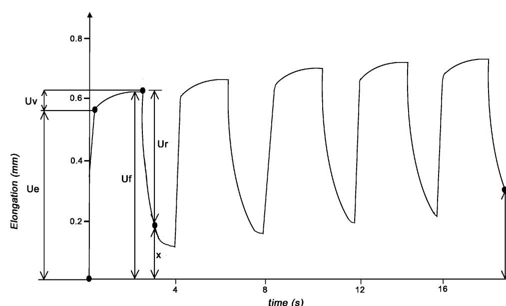

Figure 4 Cycle of five deformations of the skin measured with the Cutometer®.

Histochemical study of human skin biopsies

The tests performed with *Tripeptide-10 Citrulline* on the *in vitro* skin model were replicated *in vivo*, using a panel of three volunteers. Skin biopsies of three patients aged 33, 55 and 65, were evaluated before and after a 2-month treatment with a cosmetic formulation (twice daily) containing liposomed 0.01% *Tripeptide-10 Citrulline*. A 2-mm punch biopsy was performed on the face (between the cheek and the ear area). The collagen fibril diameter was measured from transmission electron micrographs using the AXIOVISION AC software (Carl Zeiss, Göttingen, Deutschland).

Measurement of skin biomechanical properties: skin suppleness

Skin suppleness (flexibility) has been correlated in the scientific literature to a decrease in fibril diameter [7], a function performed *in vivo* by decorin. For

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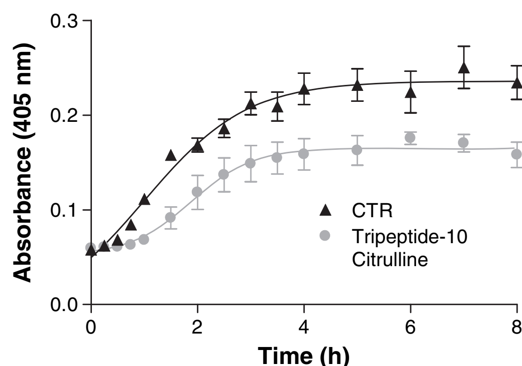

Figure 5 Effect of *Tripeptide-10 Citrulline* at 0.1% on type I collagen fibril formation.

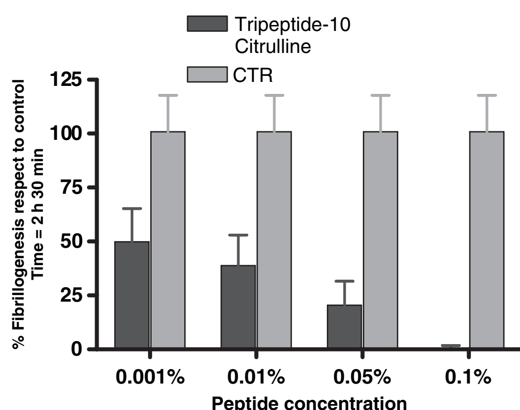

Figure 6 Dose–response effect of *Tripeptide-10 Citrulline* represents absorbance readings as a percentage of the control at 2.5 h (the time point previous to saturation). All tested concentrations of *Tripeptide-10 Citrulline* present significant inhibitory activity on fibrillogenesis respect to control, in a dose-dependent manner.

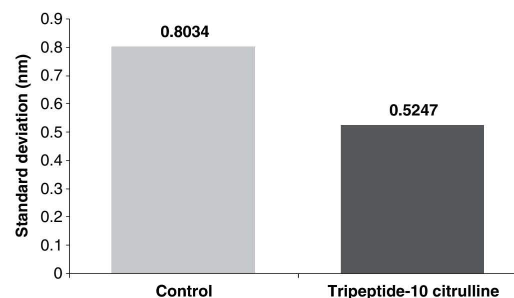

Figure 8 Standard deviation of collagen fibril diameter measurements.

this reason, skin suppleness was evaluated in a single-blind study on a group of 22 female volunteers, aged 40–58, treated with *Tripeptide-10 Citrulline*. A cream containing 0.01% of the liposomed tetrapeptide was applied daily on the face (temple) during 28 days. Another group of 21 female volunteers was treated with a placebo cream. The test was performed at an external contract research organisation (CRO).

Variations on skin suppleness were measured using an MPA 580 Cutometer. The technique consists of skin aspiration by a measurement probe. The skin is sucked into the orifice of a probe by constant vacuum pressure for a set length of time. The depth to which the skin penetrates into the probe is measured by two optical prisms located at the opening of the probe's orifice to eliminate the effects of friction and mechanical strain. Parameters are obtained after five skin aspirations. This procedure enables the evaluation of variations in the biological extensibility and elasticity of superficial cutaneous layers.

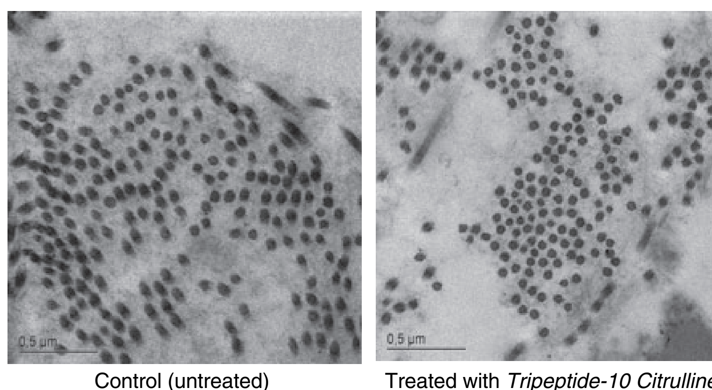

Figure 7 Transmission electron micrographs of human skin model sections from which fibril diameters were measured.

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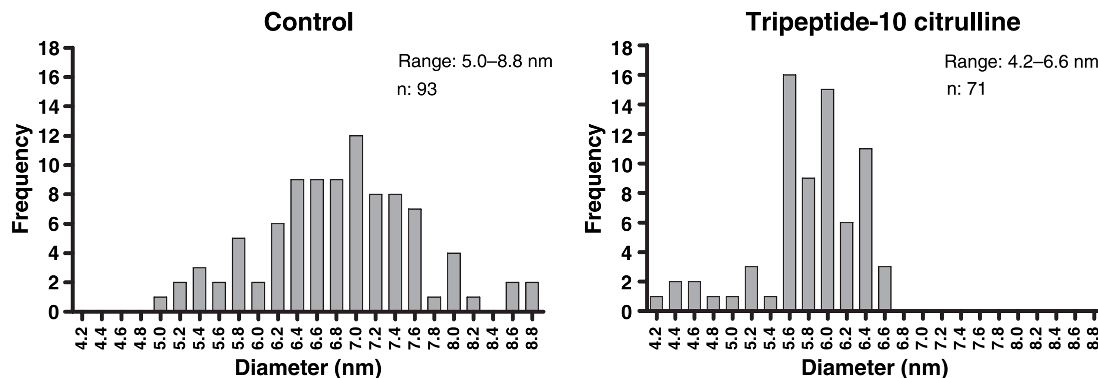

Figure 9 Distribution of collagen fibril diameter from organotypic untreated cultures (negative control) or cultures treated with *Tripeptide-10 Citrulline* 0.01%.

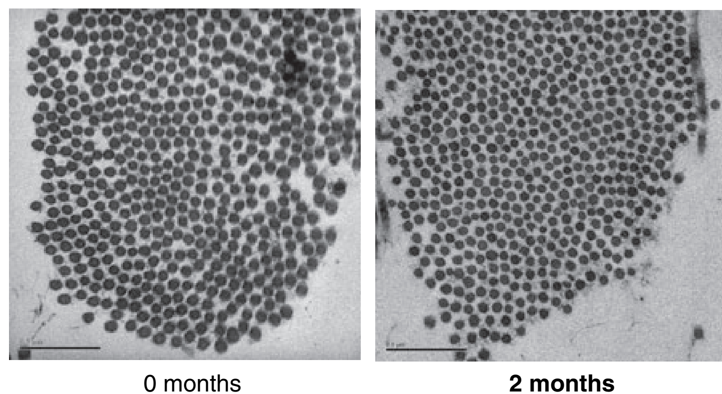

Figure 10 Transmission electron micrographs of dermal collagen from skin biopsies of 'patient 1'.

The rheological parameters obtained after five skin aspirations are (Fig. 4):

- immediate distention: Ue (mm)
- delayed distention: Uv (mm)
- total elongation Uf = Ue + Uv (mm)
- immediate retraction Ur (mm)
- residual distention after first cycle: X (mm)
- residual distention after fifth cycle: X′ (mm)

Skin suppleness is defined as the immediate skin extensibility (Ue): if the immediate extensibility increases, skin is suppler (ΔUe). Variations in skin suppleness were measured at time 0 and after 28 days.

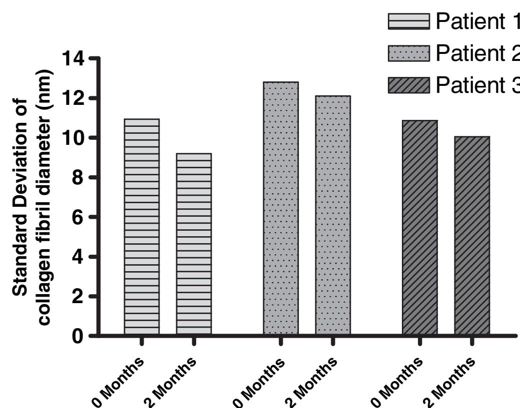

Figure 11 Standard deviation of collagen fibril diameter in skin biopsies before and after a treatment with *Tripeptide-10 Citrulline.*

Results

Collagen fibrillogenesis modulation

Twenty-three tetrapeptides were monitored in terms of their ability to inhibit the aggregation of collagen into fibrils. Six showed no effect, eight slowed down fibril formation, seven inhibited fibril formation and two promoted fibril aggregation. As decorin inhibits fibril formation [8] only, the seven peptides that inhibited fibril formation were considered. One candidate,

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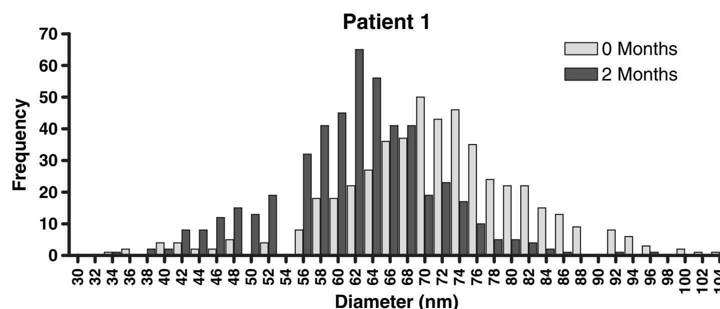

Figure 12 Distribution of collagen fibril diameter from skin biopsies of 'patient 1' before and after a 2-month treatment with *Tripeptide-10 Citrulline.*

Tripeptide-10 Citrulline, was selected according to the kinetics and dose–response results (Figs 5 and 6).

Study of collagen fibril diameter in a skin model

The diameter of collagen fibres of two areas randomly chosen from each sample was measured from transmission electron micrographs using the AXIOVISION AC software (Carl Zeiss, Göttingen, Deutschland) (Fig. 7). The data obtained from the measurements were statistically analysed using the one-way ANOVA analysis.

The standard deviation is 34.7% lower after the treatment with *Tripeptide-10 Citrulline* 0.01% (Fig. 8). The analysis of variances shows that the fibres treated with *Tripeptide-10 Citrulline* are more uniform, showing less variability (Fig. 9). There is also a decrease in fibril diameter, which is consistent with previous results [9].

Histochemical study of human skin biopsies

Results demonstrated that the average collagen fibril diameter significantly varies before and after *Tripeptide-10 Citrulline* treatment. After a 2-month treatment, collagen fibrils in all patients show a decrease in the standard deviation of the collagen fibril diameter (Fig. 10).

Analysis of frequency and distribution of collagen fibril diameter reveals that the range and distribution is different before and after treatment with a formulation containing 0.01% *Tripeptide-10 Citrulline* (Fig. 11).

All three patients show a decrease in the standard deviation of collagen fibril diameter after 2 months of treatment with *Tripeptide-10 Citrulline* (Fig. 12). This reduction implies a decrease in the variability of the collagen fibril diameters and a higher uniformity of collagen fibrils after the treatment with the peptide. The average decrease in the standard deviation of collagen fibril diameter was 9.63%.

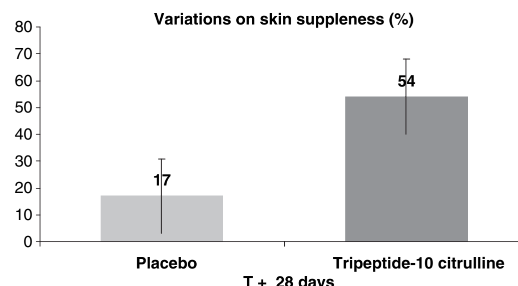

Figure 13 Increasein skin suppleness after a 2-month treatment with *Tripeptide-10 Citrulline.*

Measurement of skin biomechanical properties: skin suppleness

After 28 days, the cream containing *Tripeptide-10 Citrulline* induced a 54% increase in skin suppleness ($P < 0.001$), and this effect was observed in 95% of the volunteers (Fig. 13). No significant increase was observed for the placebo cream.

Conclusions

To sum up, the selected tetrapeptide is able to mimic decorin activity and interact with collagen fibrils, regulating the fibrillogenesis process, controlling fibril dimensions and diameter uniformity, thus helping to establish and maintain skin mechanical properties and morphology.

Acknowledgements

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