Skin Filling and Firming Activity of a Hyaluronic Acid Inducing Synthetic Tripeptide

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Accepted: 15 February 2019 / Published online: 27 February 2019
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Abstract

Aging of skin manifests in loss of volume and firming due to degradation of extracellular matrix components such as collagen and hyaluronic acid leading to wrinkling and sagging. To counteract loss of facial volume and regain firmness, fillers like hyaluronic acid (HA) are commonplace in cosmetic dermatology. We developed a synthetic tripeptide tetradecyl aminobutyrylvalylaminobutyric acid urea trifluoroacetate with proven hyaluronic acid stimulating activity in vitro. This study investigated the filling and firming activity of the tripeptide. In vitro: Microtissue technology was used to construct spherical 3D-skin equivalents. These were exposed to a tripeptide solution and content of hyaluronic acid and its receptor CD44 were assessed using histological techniques. Skin tissue culture was used to assess HA expression ex vivo. A placebo controlled, randomized, in parallel groups study to assess the firming, filling, and moisturizing activity of the peptide product was designed. We recruited 30 female Caucasian volunteers age 40 to 60 per group. Product application was twice daily for 29 days. Skin volume, deformation and moisturization were measured. HA and CD44 content in skin were increased in vitro and ex vivo. In vivo, skin firming was improved by a significant decrease in cheek deformation, a significantly restored skin volume below the eyes, and significantly improved skin hydration as measured on the cheekbone. We show evidence that the tripeptide tetradecyl-diaminobutyrylvalyldiaminobutyric urea trifluoroacetate restores facial skin volume by stimulating HA synthesis. These results underline the anti-aging activity of this synthetic tripeptide.

Keywords Peptide · Hyaluronic acid · Skin · Aging · Firming

Introduction

Skin, the largest organ of the human body, is our boundary to the environment. Skin aging occurs through intrinsic as well as extrinsic stimuli. These stimuli were recently summarized as the skin aging exposome (Krutmann et al. 2017). Facial skin aging manifests in molecular and cellular changes leading to visible phenotypic signs. Loss of collagen, HA or elastic fibers, and therefore degradation of the skin's extracellular matrix (ECM) ends up in wrinkles and sagging

skin. The accumulation of advanced glycation end products (AGEs), lipofuscin or similar changes in protein structure lead to dull looking, yellowish skin. Along those lines, skin becomes freckled with an uneven skin tone due to mottled hyperpigmentation. In addition, aged skin does suffer from dryness (Hashizume 2004), often connected to subclinical, chronic inflammation, so called inflammaging (Franceschi et al. 2007) as well as loss of volume and elasticity.

One of the main results of age-dependent ECM degradation is the loss of HA (Oh et al. 2011). HA is the main extracellular matrix component in skin. It is present in both epidermis and dermis, and comes as a high molecular weight (up to 10^5 to 10^7 dalton), linear non-sulfated glycosaminoglycan with a single polysaccharide chain (Cowman and Matsuoka 2005). It is both freely available in extracellular space but also binds to cells and many proteins containing a hyaluronic acid binding domain forming pericellular coats (Anderegge et al. 2014). One such protein is CD44, a proteoglycan, and HA's main receptor in skin (Tzello et al.

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2009). Like HA itself, CD44 is down-regulated in aged skin (Tzellos et al. 2011). Furthermore besides its function as a structural component of the ECM and regulator of diverse cellular mechanisms such as proliferation and differentiation, HA also provides the skin with moisture by binding several times its weight in water (Lee et al. 2016).

In aesthetic dermatology, HA has been widely used by injection as a filler to restore age-associated loss of facial skin volume (Gold 2007). Loss of skin volume is a hallmark of aging and results from age-dependent ECM degradation, namely HA and collagen. Its visible features comprise wrinkling and sagging but also increased facial vasculature and visible changes in pigmentation. This can be seen as an effect of skin atrophy or a thinning of the skin (Barnes et al. 2015) leading to skin losing its firmness, giving way to gravity and hanging down on the facial bones. Dermal HA fillers are high on demand and can restore skin volume like no other treatment can do (Brandt and Cazzaniga 2008). Several non-invasive treatments are available to the cosmetic dermatologist, however, despite termed non-invasive, such fillers still need injection via a needle. As many people go to lengths to turn back time and improve facial appearance topical treatments provide an alternative to injections. Currently most of them comprise anti-aging creams containing HA as one of the active components. For such solutions, HA comes as high or low molecular weight species. Another, promising path to restore facial volume is to stimulate the skin's own HA synthesis. To this end, a potential solution exists in the tripeptide tetradecyl-diaminobutyrylvalyldiaminobutyric urea trifluoroacetate (tetradecyl-DabValDab). This tripeptide was shown to stimulate HA synthesis in vitro both in dermal fibroblasts and in keratinocytes (Gempeler et al. 2018; Heidl and Gräub 2010). In a three months clinical study on female Caucasian volunteers, skin elasticity on the cheek and facial contour on the double-chin was improved (Heidl and Gräub 2010). A more recent study included 0.0005% tetradecyl-DabValDab in a cosmetic formulation together with other anti-aging actives such as carnosine, sodium hyaluronate, and *Altermonas* ferment extract (Garre et al. 2017). The authors found improved skin firmness and hydration as well as improved skin complexion based on spots and texture (Garre et al. 2017). However, although providing promising evidence on the anti-aging and facial contouring and firming activity, this study did not evaluate the tripeptide as the only anti-aging active and the study was not placebo controlled.

In the present study we set out to investigate the skin firming and filling activity of tetradecyl-DabValDab as the single cosmetic active in a placebo-controlled study on female Caucasian volunteers.

Materials and Methods

Ex Vivo Skin Tissue

Abdominal skin sample from a Caucasian female donor age 46 was harvested after obtaining informed consent and following Helsinki declaration. The tissue sample was cut in pieces of approx. 8×3 mm ($\varnothing \times$ thickness) and cultured in an air–liquid interface in a perforated ring of stainless steel in contact with culture medium (modified Williams' E medium) up to day 6. Eight skin specimens were used for each treatment. Two skin samples were cultured to perform the skin viability test and six to perform marker analysis. The culture medium was renewed every three days. Both the test samples and the controls were applied topically and renewed daily. The application has been performed as follows: skin biopsies have been gently cleaned with a cotton pad and per treatment 4 μ l of tested sample in DMSO has been applied on top of each piece and covered with a 6 $\varnothing$ mm delivery membrane (3M). Twelve skin sections for each treatment were stained by alcian blue (Alcian Blue 8GX staining kit, Sigma–Aldrich #A3157) that dyes acid mucins, such as hyaluronan, in blue. The amount of HA present in each slide was evaluated by estimating the intensity and the distribution of blue within a selected area of the dermis using Image J (NIH-USA). The obtained value has been normalized upon the considered area. A paired Student's t-test was used for statistical analysis.

Skin Microtissues

Spherical 3-dimensional skin microtissues (3D InSight Skin Microtissues, InSphero AG, Schlieren, Switzerland, MT-07-001-01; Lot hSkMT014) were made as per the manufacturer's instructions. They consist of a dermal core surrounded by an epidermal outer layer. Tissues were kept in a 96 well plate (Akura 96-well plate, InSphero AG, Schlieren, Switzerland) in culture medium for 10 days until harvest. Microtissue compound treatments took place at days 0, 2, 5, and 7. After harvest, skin microtissues were paraffin embedded and cut into 6 μ m tissue sections. Hyaluronic acid was stained with alcian blue (Chroma 8GS, AppliChem GmbH, Germany) according to standard histochemical procedures. Nuclear fast red-aluminium sulfate solution (Waldeck, GmbH, Germany) was used to counterstain cell nuclei. CD44 immunofluorescence-staining: Tissue section from skin microtissues were processed according to standard histology procedure. A mouse anti human CD44 (Novocastra, Clone DF1485) at dilution 1:200 and a donkey anti-mouse Alexa488 (Jackson Immune Research)

at dilution 1:5000 was used for primary and secondary staining respectively. CD44 imaging and quantification: Stained skin microtissue sections were assessed with a Leica DMi8 FL microscope (Leica, Germany) and images were captured with a DFC9000 GT fluorescence camera (Leica, Germany) at 40x magnification at 300 ms exposure time. Image based quantification was performed with ImageJ software (NIH, Bethesda, U.S.). Total tissue area was assessed for total fluorescence intensity. To enable comparison of staining intensities in between samples, unstained non-tissue areas of the individual images were assessed and used for subtraction of background signal.

Clinical Study

The clinical study was performed by Institute proDERM, an independent clinical research organization in Schenefeld/Hamburg, Germany, in the fall 2017. Thirty-three subjects per group were recruited for this placebo controlled, randomized, parallel-groups study. They were all female Caucasian between age 40 and 60 (mean 52.5 years for placebo group, mean 52.4 for active group) with self-perceived sagging of the face and mild to moderate photoaging according to Griffith's scale 2–6. Volunteers included pre- as well as post-menopausal volunteers but excluded pregnant subjects and subjects with a BMI > 30, and a weight of > 100 kg. Volunteers were instructed not to change their dietary habits during the study. Otherwise standard inclusion and exclusion criteria were used. Subjects gave their informed consent to participate in the study, the declaration principles of Helsinki and good clinical practice (GCP) were applied.

The weather conditions in Schenefeld/Hamburg during the study showed maximal daily temperatures between 17.4 and 5.4 °C, and minimal daily temperatures between 11.5 and – 1.7 °C, and relative humidity between 68.67 and 98.75%. Subjects were instructed to use twice daily 0.5–2 mg/cm² of a cream containing a base formulation or the active formulation (Table 1) on full face. The instrumental measurements took place in an air-conditioned room at a temperature of 21 ± 1 °C and at 50 ± 5% relative humidity. Before measurements, the subjects stayed in the climatized room for at least 30 min. Adverse reactions were documented.

Clinical Test Methods

Skin firmness was assessed using the DynaSKIN device by Eotech SA, Marcoussis, France (Kearney et al. 2017). In this method an air stream is blown perpendicularly onto a defined spot on the cheeks of the volunteers causing a skin deformation. The greater the deformation, the less resistant the skin is against the pressure of the air stream, meaning the less firm the skin is. A 3D-camera records this deformation. From this the three parameters negative volume (mm³), depth (mm) and circumference (mm) of the deformation are calculated. A decrease of any of these three parameters indicates a firming effect because the skin is more resistant to the pressure of the air stream. To visualize the average change of the skin deformation over time we generated mean deformations per group and timepoint. Measurements were taken on both sides of the face and average values were calculated. Of the 3D-images showing the depth and the circumference of

Table 1 Formulations used in clinical study

Component (INCI)	Active formulation (% w/w)	Placebo (% w/w)
Stearyl alcohol	2.5	2.5
Octocrylene	8	8
Butylmethoxydibenzoylmethane	3	3
Polysilicone-15	0.9	0.9
Ethylhexylsalicylate	4	4
C12-15 alkylbenzoate	3	3
Coco-caprylate	3	3
Dimethicone	2	2
Potassium cetylphosphate	2	2
Hydroxyethylacrylate/Sodium acryloyldimethyl taurate copolymer	0.8	0.8
Aqua	64	66.5
Disodium EDTA	0.1	0.1
Butylene glycol	3	3
Xanthan Gum	0.2	0.2
Phenoxyethanol, Ethylhexylglycerin	1	1
Glycerin, Aqua, Magnesium chloride, Tetradecyl Aminobutyrolylvalylamino butyric urea trifluoroacetate	2.5	0

the deformations by a color code, we made mean images of one facial side, group, and timepoint.

Skin volume was measured by firstly acquiring 3D-image scans of the volunteer's faces by the AEVA-HE device (Eotech SA, Marcoussis, France). From the resulting, computer generated 3D-images of the face one can calculate changes in volume at various areas of the face as well as differences in distance towards the image acquisition system over time. In particular, we manually defined a region of interest (ROI) for the area below the eyes for timepoint day 0 (Fig. 1). This area is a target for hyaluronic acid fillers as it becomes hollow with age. This region is automatically registered at other time points thanks to a specific registration algorithm. The volume is defined as the volume of the convex hull of the ROI. The convex hull is the smallest closed and convex volume that completely contains a surface (Fig. 1). To measure the distances between the surface at baseline and after treatment, the same ROI as the one defined for the volume analysis is applied on the distance

Fig. 1 Segmentation of region of interest (ROI) at area below the eye. A 3D-face scan taken by the AEVA-HE system is shown on the right. A blow-up of the eye area is shown with the ROI in blue on the left. (Color figure online)

Fig. 2 Stimulation of HA content by the tripeptide ex vivo. **a** bar graph showing the content of HA in the skin sections relative to vehicle. Error bars represent standard error of the mean. **b** Representative

map. The distance is defined as the mean value of the distances for each pixel over the ROI (Fig. 1). Ideally, volume and distance point into the same direction. We then calculated a color gradient displaying distance to visualize changes.

Skin hydration was measured on the cheekbone. Five measurements per facial side, all in a row on the cheekbone were recorded using a Corneometer CM 825 (Courage & Khazaka Electronic GmbH, Cologne, Germany) and mean values calculated. This device measures stratum corneum hydration by electrical capacitance.

Results

Tetradecyl-DabValDab Increases HA and CD44 in Skin Tissue

We showed previously that tetradecyl-DabValDab could stimulate HA both in dermal fibroblast and epidermal keratinocytes (Heidl and Gräub 2010). We were interested if the tripeptide was able to stimulate hyaluronic acid in skin ex vivo. We found an increased HA content (+ 56%) in skin tissue after topical incubation with the tripeptide (Fig. 2a). This is also seen in representative skin sections showing an increase in blue HA staining mainly in the papillary dermis (Fig. 2b). Retinoic acid was used as anti-aging reference molecule but did not show an increase in hyaluronic acid stimulation (Fig. 2).

As one of the main HA receptors in skin is CD44 and it was shown to be down-regulated in aged skin (Tzellos et al. 2011), we were interested, if the tripeptide could rescue CD44 expression in a similar way as HA expression. We used skin microtissues consisting of a dermal core comprising dermal fibroblasts and a stratified outer layer of

skin sections stained with alcian blue to display HA. HA is mainly detected in the papillary dermis (arrows). With 20 ppm of tripeptide a signal is also detected in the reticular dermis (arrowhead)

keratinocytes representing the epidermis. We first assessed HA expression using alcian blue staining after incubation of the microtissues with the tripeptide product. Although we failed to quantify HA expression we found increased alcian blue staining in sections of the microtissues (not shown) in line with our *ex vivo* results (Fig. 2). We then assessed CD44 expression. We found an increase of 32% in CD44 staining similar to incubation with basic fibroblast growth factor (bFGF) (Fig. 3), a main stimulator of HA in skin (Heldin et al. 1989).

Skin Firmness on the Cheeks is Increased by a Formulation Containing the Tripeptide

A decrease in skin deformation evoked by a perpendicular airstream representing increased skin firmness on the cheeks was recorded for all three parameters, negative volume of the deformation, circumference of the deformation and depth of the deformation. For negative volume we measured a decrease of 5.45 mm³ after 29 days ($p < 0.05$) (Fig. 4a), and for circumference we measured a decrease of 3.54 mm after 29 days ($p < 0.05$) (Fig. 4b). The average

depth of the deformation also decreased by 0.006 mm after 29 days (Fig. 4c). Mean images representing the deformation by a color code also show the decrease in circumference after 29 days application of the active formulation (Fig. 4d). Although we also recorded a small firming effect for the placebo group it was not significant versus baseline. In addition, for both negative volume and depth (Fig. 4a, b) the effect seen at day 15 for the placebo group was decreased at day 29, whereas for the active group all three parameters showed a time-dependent improvement.

The Active Formulation Restores Volume at the Area Below the Eyes

The area below the eye becomes hollow with age leading to decreased firmness resulting in sagging eye bags. A restoration of volume with hyaluronic acid counteracting dermal atrophy can relieve the hollowness and sagging. We found an increase in skin volume both with looking at distance change over time (+0.11 mm at day 15 and +0.14 mm at day 29, both $p < 0.05$ vs. baseline) (Fig. 5a), as well as for volume under the ROI (+20.3 mm³ at day 15 and +27.4 mm³ at

Fig. 3 The product containing the tripeptide increases the expression of CD44 in skin microtissues (orange bar). The images above show representative sections of the skin microtissues with the green staining of CD44 at day 10 of incubation

Fig. 4 A formulation containing the tripeptide leads to an overall decrease of the skin deformation evoked by the DynaSKIN device, as measured by volume, circumference and depth, meaning it increases skin firmness on the cheek. **a** Negative volume of the skin deformation is decreased at days 15 and 29 compared to baseline indicating firmer skin. * $p < 0.05$ versus baseline. **b** Circumference of the skin

deformation is decreased at days 15 and 29 indicating firmer skin. * $p < 0.05$ versus baseline. **c** Depth of the skin deformation is decreased at days 15 and 29 indicating firmer skin. **d** Mean images of the skin deformation in false color to display depth and circumference. At day 29 the circumference is visibly decreased at the cheeks of volunteers using the active formulation

day 29) (Fig. 5b). This is also shown using a color gradient displaying volume changes over time (Fig. 5c).

Increase of Facial Hydration

The hydration of the stratum corneum measured via capacitance on the cheekbone showed a significant increase of 4 units after 29 days ($p < 0.05$ vs. placebo, $p < 0.001$ vs. baseline) that was not seen in the placebo group (Fig. 6).

Discussion

During aging skin loses volume due to a decrease in extracellular matrix components such as collagen and HA (Gold 2007). This causes decreased firmness and elasticity leading to skin sagging. We developed a synthetic tripeptide tetradecyl-DabValDab capable of inducing the skin's own HA synthesis in vitro. The aim of this study was to assess the potential of the tripeptide to counteract age-dependent cutaneous volume loss and hence restore skin firmness. We could show that the tripeptide was able to induce HA synthesis ex vivo and in three-dimensional skin equivalents, so called skin microtissues where the main HA receptor CD44 was

induced, too (Figs. 2, 3). These results were in line with previous findings in dermal fibroblast cell culture and keratinocyte cell culture showing a significant stimulation of HA synthesis (Heidl and Gräub 2010). The lack of significance could come from a decreased bioavailability of the tripeptide in complex culture systems, or a decreased activity due to counteracting mechanisms and biological feedback loops. However, a significant HA-stimulating activity ex vivo was found in combination with UV-irradiation (Gempeler et al. 2018) supporting the findings presented here. Concerning the clinical study, we found an increase in skin firmness on the cheeks (Fig. 4). We propose that this increase in skin firmness resulted from an increase in the skin's HA content after treatment with a tripeptide containing formulation. Injections with an HA-filler showed indeed a positive effect on skin elasticity before (Reuther et al. 2010). In addition, our previous in vitro results found an increase in collagen I and the proteoglycans lumican and decorin (Heidl and Gräub 2010), as well as on ex vivo skin an increase in collagen VII which is part of the basal membrane anchoring fibrils (our own unpublished data). These effects may as well contribute to the skin firming effect found in vivo. Our results are also in line with an increase in elasticity found previously with this tripeptide using the cutometer device (Heidl and Gräub

Fig. 5 Skin volume below the eyes is restored after using the active containing formulation. **a** Skin volume is time-dependently restored with the active formulation (orange bars) compared to the placebo group. **b** Skin surface distance to baseline is increased compared to placebo indicating restoration of skin volume * $p < 0.05$ versus placebo. **c** Representative images showing skin volume restoration below

the eyes. 3D-image scans for volunteers 23 (placebo group) and 58 (active group) are shown. Image blown-ups displaying a volume gradient per pixel on the skin show gradual restoration of volume (yellow to orange color). In volunteer 58 the distribution of the orange color is more even and faster

2010). Furthermore, Garre et al. showed a similar result as the one presented here after treatment with a formulation containing several anti-aging actives including the tripeptide used here (Garre et al. 2017). Their results were even more significant which could account for the various additional anti-aging actives used in their formulation. As the tripeptide induces HA synthesis in skin one could assume that the overall skin volume is increased after application of the tripeptide. We measured this on the area below the eyes, an area prone to hollowness and sagging in aged skin. We used three dimensional scans taken with the AEVA-HE device. We calculated the volume under the ROI and the distance of the skin at various timepoints compared to baseline for each pixel in the ROI. Both methods revealed an

increase in volume (Fig. 5). This is underpinned by the finding that hydration on the cheekbone also increased significantly (Fig. 6). We again propose that this increase in hydration is the result of an increase in cutaneous HA content. In fact, it was found that injection of HA at various facial sites increased facial skin hydration as measured by corneometer (Seok et al. 2016). Moreover, an increase in hydration would presumably also result in smoothing of fine lines and a small increase in volume. Looking at the whole face the increase in skin volume around the cheeks, the cheekbone and the eye area could have a lifting effect on the lower parts of the face. Indeed, in our previous study we found a decrease in neck contour (Heidl and Gräub 2010) supporting this hypothesis. In addition, an evaluation with several

Fig. 6 Skin hydration on the cheekbone is increased on volunteers using the active formulation (orange bar). ** $p < 0.01$ versus baseline, * $p < 0.05$ versus placebo

hyaluronic acid treatments showed increases in skin volume on the cheekbone going along with decreased sagging (Nobile et al. 2014). In conclusion, our data provide further evidence for the anti-aging and facial contour remodeling activity of the tripeptide tetradecyl aminobutyrylvalylaminobutyric acid urea trifluoroacetate, and that this activity was due to stimulation of the skin's own HA synthesis.

Acknowledgements We are grateful to the volunteers of the clinical study for their participation. We thank our colleagues Eliane Wandeler and Dominik Imfeld (DSM Nutritional Products) for fruitful discussions during the project. The tripeptide tetradecyl-diaminobutyrylvalyldiaminobutyric urea trifluoroacetate is marketed by DSM Nutritional Products under the trade name SYN®-HYCAN.

Funding This study was funded by DSM Nutritional Products Ltd. RC, EJ, GL and MG are employees of DSM Nutritional Products Ltd.

Compliance with Ethical Standards

Conflict of interest All other authors declare no conflict of interest.

Ethical Approval All procedures performed in this study involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments. Written informed consent was obtained from all individual participants included in the study. The human study was done with a cosmetic product already on the market using topical application. Cosmetics have to be safe products. European legislation has therefore produced positive and negative lists for the constituents of cosmetics. Cosmetic products manufactured according to cosmetic Good Manufacturing Practice (Cosmetic GMP) can be considered per se as safe for human testing. Therefore, no individual review by an ethics committee was deemed necessary for this study.

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