AMINO ACID AND HYALURONIC ACID MIXTURES DIFFERENTIALLY REGULATE EXTRACELLULAR MATRIX GENES IN CULTURED HUMAN FIBROBLASTS

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The aim of this screening study was to evaluate the efficacy of different proprietary mixtures of amino acid and hyaluronic acid (HA) in stimulating the production of extracellular matrix (ECM) components, particularly the neo-synthesis of elastin, and in promoting a more efficient deposition of elastic fibres (elastogenesis), while at the same time maintaining the stimulation of collagen. The study has allowed identification of the optimal ratios between the amino acids (AA) for the production of collagen and elastin. Human primary dermal fibroblasts from a 44-year-old female donor were used as a test system in an experimental design based on the evaluation of the expression of relevant ECM genes using a transcriptomic dynamic approach. The expression of ECM genes was evaluated by RTqPCR from 24 to 120 hours in the presence of the test items. Moreover, the production of ECM proteins was verified by Western blot analysis after a 120 h treatment period. In addition to elastin, collagen IV, a fundamental structural component of the basal lamina responsible for epithelial and connective tissue anchoring, was analysed as potential target for the modulation of ECM protein production by human fibroblast. The first phase of the study demonstrated that alanine and valine are essential to promote production of elastin, of which they are important constituents. The second phase of the study, which was conducted to clarify the interactions between the different clusters of AA, demonstrated that it is necessary to choose a mixture that contains specific amounts of amino acids of both proteins, collagen and elastin, to give a significant response and a significant production of both. This also proves the existence of a ratio between the 2 clusters (AA elastin/AA collagen) that guarantees an adequate and balanced response to gene expression and production by fibroblasts, collagen and elastin. The study has allowed identification of the optimal ratios between the AA for the production of collagen and elastin.

The use of biomaterials, a broad category of materials that also comprises biopolymers for clinical application, is becoming increasingly important and hyaluronic acid (HA), in particular, is one of the most used. Due to its unique physical characteristics (hygroscopicity and visco-elasticity), it is largely employed in several fields of medicine including: ophthalmology, dentistry, rheumatology and regenerative and aesthetic medicine (1-5). In addition to its mechanical and physical properties, HA has important biological functions, binding specific membrane receptors (6), regulating cellular adhesion, proliferation and migration, cytokine synthesis and deposition of extra cellular matrix (ECM) proteins (7-8).

It has been demonstrated that adding small molecules, such as mixtures of amino acids (AA) (e.g. proline, lysine, glycine and leucine), to HA can enhance its action in many fundamental biological processes, and mainly in wound healing (9-10).

Key words: amino acids, fibroblasts, elastogenesis, elastin, collagen

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The biological wound healing process is one the most complicated processes described in relation to the skin and involves different and overlapping phases such as inflammation, proliferation, migration of keratinocytes, new tissue formation and tissue remodelling: all these steps are necessary to achieve tissue regeneration and wound healing (9).

All these phases are characterized by biochemical, molecular and morphological changes and even if not entirely characterized, they can be monitored in the different skin compartments thanks to identification of the relevant bio-markers. During the latter phases (re-modelling), ECM proteins, which are massively synthetized, assembled and reorganized by fibroblasts allowing tissue reconstruction, play a critical role (11).

These proteins include collagens and fibronectin, the biosynthesis of which is strictly connected to the local bioavailability of constituent AA. Collagen triple helix consists of glycine, L-proline and L-lysine in well-determined positions. Following the activation of specific hydroxylases (e.g. LOXL1) by L-lysine, the hydroxylation of glycine and proline residues causes the formation of inter- and intra-molecular bonds, which give collagen its viscoelastic properties (12). It has been shown that the use of an AA-enriched HA (Aminogam®) promotes the expression of type I and III collagen, the major components of connective and granulation tissues respectively, and of fibronectin genes in human pulmonary fibroblasts (13). Moreover, the expression of ECM deposition regulators [such as TGFβ and, connective tissue growth factor (CTGF)] has been shown to be increased by the treatment (13).

Recently, attention has been paid to other components of ECM in order to investigate the further effects of HA mixtures and to discover other potential clinical applications (14). Of the components of ECM, elastic fibres play a fundamental role for the structure and function of elastic tissues such as the bladder, cartilage, ligaments, blood vessels and the skin.

The elastic fibres consist of microfibrils and elastin. Microfibrils mainly consist of fibrillin, and provide a scaffold which guides the alignment and the cross-linking of elastin molecules by one or more members of the lysyl oxidase (LOX) gene family (15).

Elastin is a polymeric protein derived from the

Table I. *Amino acid composition of the tested formulations.*

		%			
		Mixture A	**Mixture B**	**Mixture C**	**Mixture D**
COLLAGEN	Glycine	50	34.8	33.4	30.2
	L-Proline	37.5	26.1	25.1	22.7
	L-Lysine HCl	5.5	3.8	3.7	3.3
	L-Leucine	7	4.9	8.7	4.2
ELASTIN	L-Valine	-	12.9	12.3	16.8
	L-Alanine	-	17.5	16.8	22.8

Table II. *TaqMan probes for genes of interest and endogenous control used in RTqPCR analysis.*

Gene	Code	Taqmas assay no.
Elastin	ELN	Hs00355783_m1
Collagen IV	COL4A1	Hs00266237_m1

crosslinking of secreted monomers of tropoelastin precursors.

Elastin has an AA composition similar to that of collagen in terms of glycine and proline residues, but with a specific enrichment of the hydrophobic residues, valine and alanine (16), therefore it can be assumed that increasing the bio-availability of these specific AAs could enhance elastin synthesis.

The aim of this screening study was to evaluate the efficacy of different proprietary mixtures of AAs and HA in stimulating the production of ECM components, particularly the neo-synthesis of elastin, and in promoting a more efficient deposition of elastic fibres (elastogenesis), while at the same time maintaining the stimulation of collagen. The study has allowed identification of the optimal ratios between the AA for the production of collagen and elastin.

For this purpose, human primary dermal fibroblasts from a 44-year-old female donor were used as a test system in an experimental design based on the evaluation of the expression of relevant ECM genes using a transcriptomic dynamic approach. The expression of ECM genes was evaluated by qRT-PC from 24 to 120 h in the presence of the test items. Moreover, the production of ECM proteins was verified by Western blot analysis after a 120-h treatment period.

In addition to elastin, collagen IV, a fundamental structural component of the basal lamina responsible for epithelial and connective tissue anchoring, was analysed as potential target for the modulation of ECM protein production by human fibroblast.

MATERIALS AND METHODS

Test items

Different proprietary formulations of AA-enriched HA (Professional Dietetics, Milan, Italy) were tested in this study. The compositions of the mixtures are reported in Table I.

Cell culture

The human dermal fibroblasts were isolated from a breast surgery resection from a 44-year-old Caucasian woman, who signed informed consent. The epidermis was separated from the dermis with overnight incubation with Dispase II. The dermis was digested with Collagenase I and fibroblasts were seeded in DMEM F12 medium with 20% fetal bovine serum and antibiotics (1% penicillin/streptomycin) and ascorbic acid P (1 mM), and then used for the assays in steps 1-3.

Selection of treatment dosage by MTT assay

The cytotoxicity of the test items was preliminarily assessed after 120 h. This time was chosen because it was the longest time to be applied in the efficacy test at which the three concentrations used were non cytotoxic. The cytotoxicity was studied at three different concentrations of 10-100-1000 μg/mL in the medium, before proceeding with the efficacy test. The fibroblasts were seeded at 10^4 cells/well in a 96-well plate and cultivated until confluence, then the test item and the negative control were applied in triplicate. After 120 h of product application, the MTT Test was performed to measure cell viability (Table II). Briefly, fibroblasts were incubated in a 0.5 mg/ml solution of MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide) (Sigma) in PBS at 37°C, 5% CO_2. After this period, the tetrazolium ring yielding blue/purple MTT crystals, which were extracted by isopropanol and optical density, was measured at 570 nm using the Tecan Infinite M-200 spectrophotometer. The results are expressed as a percentage of cell viability compared to the negative control.

Experimental design

Human fibroblasts were seeded at a density of 10^4 cells/well in a 96-well plate and cultivated until confluence. The test items were dosed in the culture medium at 1000 μg/mL and maintained throughout the treatment period. The read-out parameters were measured after 24 h, 72 h and 120 h for gene expression and at 120 h for Western blot.

Real Time Quantitative PCR

The RNAqueous method (RNAqueous kit, Ambion, Life Technologies) was used to extract the total RNA from cell samples. Each biological replicate was run in triplicate. The High Capacity cDNA Reverse Transcription kit (Life Technologies) was used to synthetize cDNA from RNA. The instrument used was the Applied Biosystems 7500 Fast Real Time PCR with fluorescent-based PCR chemistry, the TaqMan assay. TaqMan gene expression

assay and cDNA (25 ng) were added to the 2X TaqMan Fast Universal PCR Master Mix for a total volume of 20 µL. The Thermal condition steps in the ABI 7500 Fast were: 95°C 20 sec; 40 cycles (95°C 3 sec +60°C 30 sec).

GAPDH was used as an endogenous control gene to normalize input amounts. The list of TaqMan probes for genes of interest and endogenous control is reported in Table II.

RT-PCR fluorescence data, generated by the ABI 7500 Fast thermocycler, was collected by the internal SDS 2.0.6 software. Relative gene expression was calculated for each formulation compared to the negative control using the $2^{\Delta\Delta ct}$ method. The software uses a 95% confidence level to calculate errors.

A value was accepted as significant when the gene was "one-fold" up- [Relative Quantification (RQ)>2] or down-regulated (RQ<0.5) compared to the calibrator sample (RQ=1). The internal instrument confidence level used was 95%.

Table III. *Cell viability by MTT assay after 120 h treatment with products A, B, C, D at 10-100-1000 µg/mL.*

µg/mL	Viability % at 120H	sd
NC	100	0.6
A 10	97.29	1.1
B 10	96.90	1.5
C 10	103.34	11
D 10	97.42	0.8
A 100	96.25	2.0
B 100	90.62	2.3
C 100	102.81	0.8
D 100	95.22	2.6
A 1000	91.97	1.6
B 1000	92.25	0.9
C 1000	96.09	1.4
D 1000	89.92	0.6

NC: negative control (untreated cells).
Percentages of viable cells are reported.
Both treated cells and NC were tested in triplicate.

Table IV. *Relative quantification of elastic protein performed considering Negative Control (NC) at 120 h and calibrator sample.*

RELATIVE QUANTIFICATION	ELN	COL4A1
NC	1	1
A	1.478	1.484**
B	1.056	1.330*
C	2.121**	2.463**
D	2.164**	3.197**

***p<0.01 and *p<0.05 (Student t-test)*

Western blot

Fibroblast cell samples were placed in Bio-Plex Cell Lysis buffer (BIORAD) with freshly added protease inhibitor mixture (cocktail SRE 0055 Sigma (containing aebsf, aprotinin, bestatin, e-64, EDTA, leupeptin), homogenized, and stored on ice for 30 min, then centrifuged at 16,000 x g for 10 min at 4°C. Protein determination was performed with RC DC Protein Assay (BIORAD). After adding an equal volume of 2X Laemmli Sample Buffer to the lysate, each sample (10 μg of total protein) was boiled in sample buffer at 100°C for 5 min, separated on Tris-Glycine Extended (TGX) Stain-free precast gels 7.5% (Bio-Rad) and transferred to PVDF membrane (BIORAD).

The membranes were blocked for 1 h at room temperature using 3% blocking solution (BSA) in TBST 0.05%. The membranes were incubated with anti-COL4A (SantaCruz, sc-59814) or anti-ELN (Santa Cruz sc-166352) Mouse monoclonal antibody for 1 h at room temperature and then rinsed three times with TBST for 5 min each time.

Bound antibodies were detected with the recommended dilution of labelled goat anti-mouse IgG secondary antibody (Invitrogen) in TBST at room temperature for 1 h. After three washes in TBST, the membrane is ready for signal development with Immun Star Westernc Kit (Biorad).

The chemiluminescence bands of COL4A and ELN were visualized by using Chemidoc XRS system (Biorad) and quantified using ImageLab software (250 kDa and 70/55 kDa, respectively). Protein levels were normalized on total protein content.

RESULTS

The study has two evaluation phases:
- Phase one: to clarify whether the proposed mixtures could produce both collagen and elastin
- Phase two: optimization of the ratios between the various AAs

Phase One Results

Cytotoxicity test

All products induced residual cellular viability not lower than 90% for all tested concentrations (Table III).

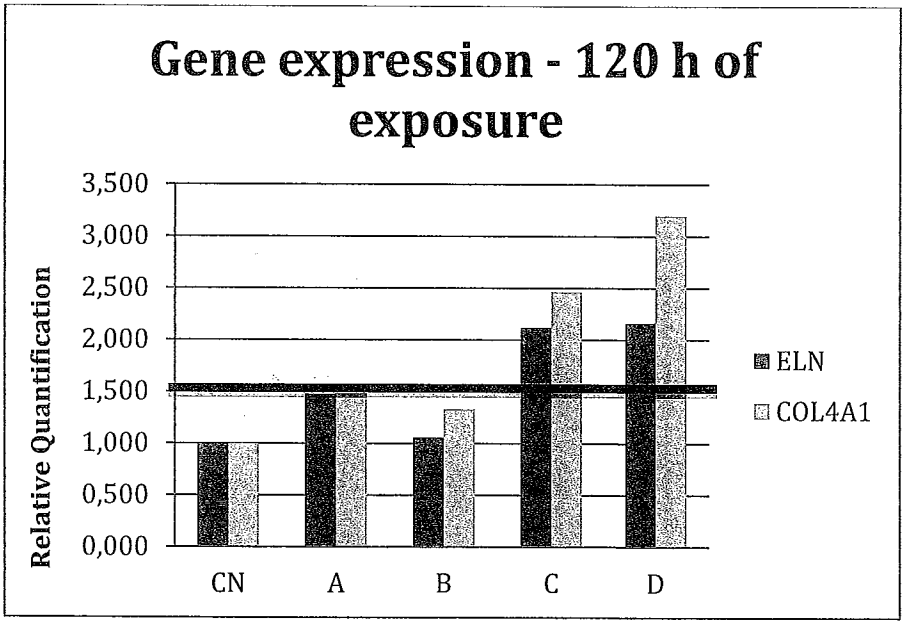

Fig. 1. *Expression of ELN, FBN-1, FN-1, LOXL-1 and COL4A1 genes in cultured fibroblasts after 120 h of treatment with different mixture of amino acids and hyaluronic acid. Negative Control (NC) was used as calibrator sample for relative quantification calculation NC=1*

Since non-toxic effects were noted, in order to maximize the potential biological response to the treatment, the higher concentration (1000μg/mL) was selected for the subsequent efficacy assays.

Transcriptional study

As expected, since mainly structural proteins were investigated, none of the genes involved in EMC formation were modulated until 72 h (data not shown). On the contrary, as reported in Fig. 1 and Table IV, a significant modulation of ELN and COLIV genes was quantified after 120-h treatment.

After 5 days of growth, compared with the negative control (untreated), mixture A, the effects of which were investigated in a number of studies, seemed not to exert any significant action on the expression of the selected genes. The addition of alanine (17.5%) and valine (12.9%) to mixture B also had no effect on the levels of the transcripts, when leucine was only 4.9%.

Otherwise, mixture C (with the highest L-leucine content 8.7%) and mixture D [with the highest L-Valine (16.8%) and L-Alanine content (22.8%)] were able to overexpress collagen IV and elastin, thus promoting the assembly of matrix components.

Protein synthesis

Western blot analysis was performed exclusively at 120 h.

Fig. 2 and Table V show the Western blot images and relative quantitative analysis of the bands for collagen IV protein. The protein was found to be significantly modulated in samples treated with mixture C and D and very close to significance in samples treated with mixture B.

Western blot images and relative quantification of elastin are shown in Fig. 3 and Table VI, respectively.

As can be seen in Fig. 3, elastin protein presented two different bands corresponding to the soluble non-cross linked precursor of elastin (tropoelastin, 70KDa) and elastin form of 55 kDa. Quantification of the single bands not only showed a different capacity of the tested mixtures to modulate elastin

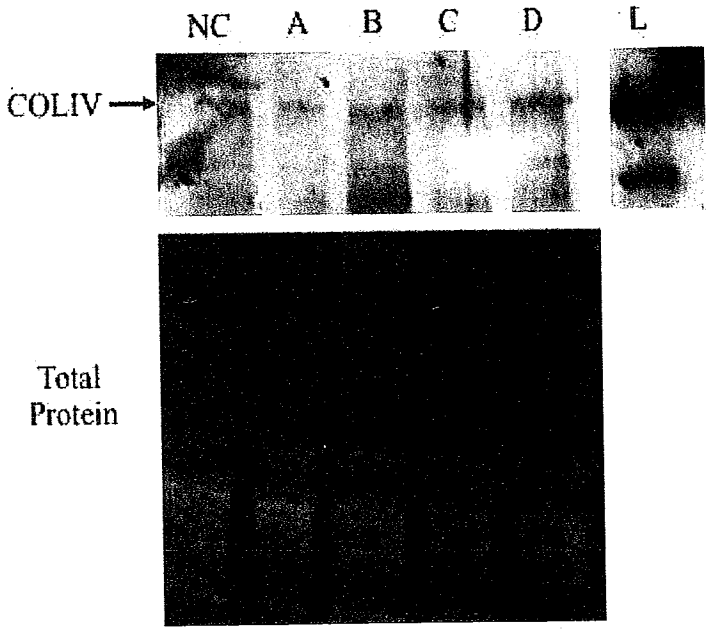

Fig. 2. *Western blot analysis of collagen IV. Lanes: NC: negative control; A-D: test items; L: molecular weight ladder*

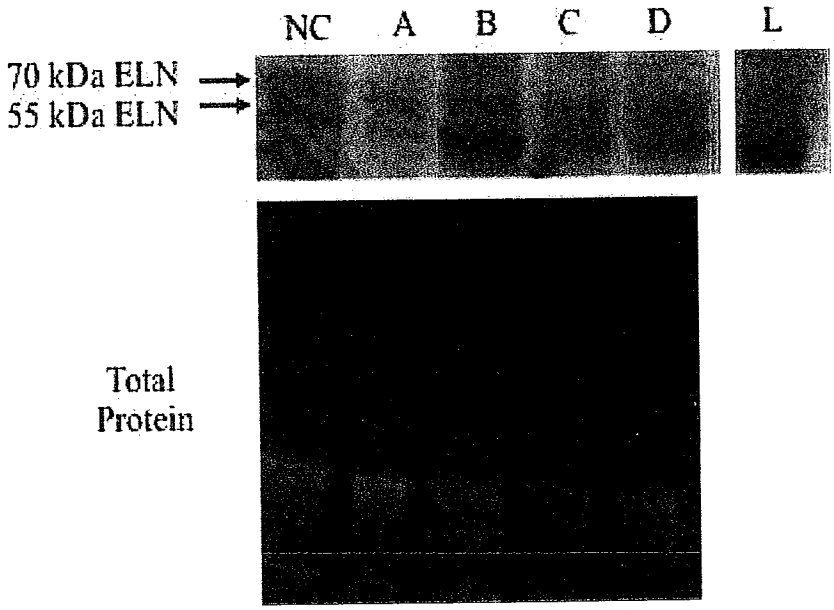

Fig. 3. *Western blot analysis of elastin. NC: negative control; A-D: Test items; L: molecular weight ladder.*

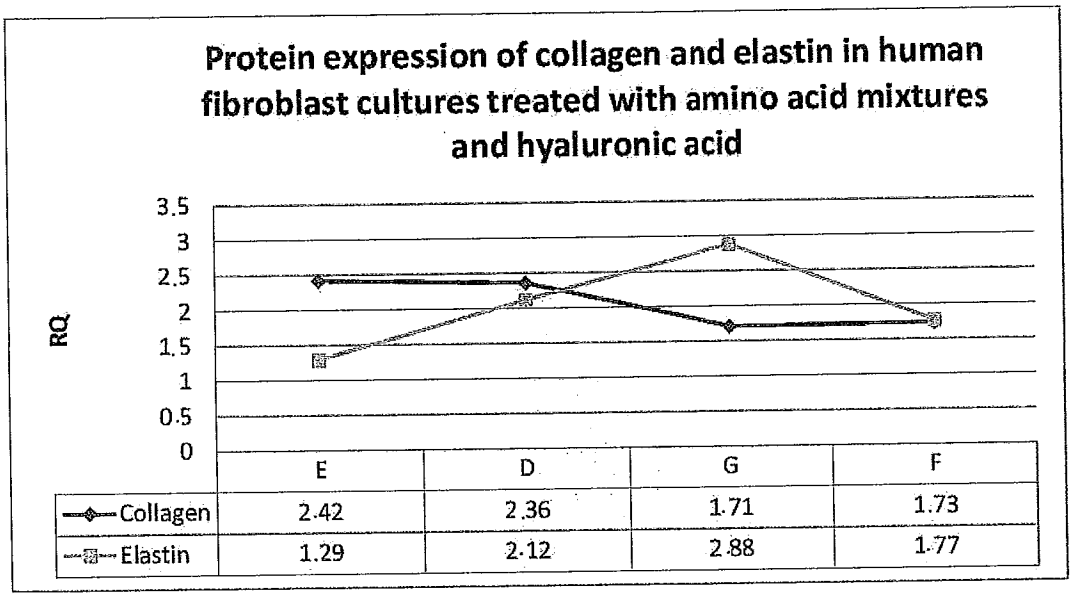

Fig. 4. *Relative quantification of collagen IV and elastin proteins performed as negative control (NC = 1) at 120 h as calibrator sample.*

production but also a differential action on the two different forms. In particular, mixture B seemed to be the most active formulation, enhancing both the forms with different intensity, mixture C increased only the insoluble α-elastin, while mixture D exerted a stimulatory action on precursor and final protein in equal manner.

Phase Two Results

In the first study it was shown that the three

different 6 AA (and not 4 AAs as in mixture A)-mixtures are able to significantly produce both collagen and elastin in respect to the control, while the 4 AA mixture is capable of stimulating the production of collagen alone.

In the second part of the study, three different AA clusters were defined to clarify the limits and potentials of the mixture table:-

- More expressed AAs in collagen (glycine, lysine, proline)
- More expressed AAs in elastin (alanine, valine)
- Leucine, essential AA, primer of protein synthesis as demonstrated in several studies (18).

In this second experimental session, all blends contained the same amount of leucine, the lowest of all those tested in the first session of the experiments, in order to more clearly evaluate the effectiveness of the other clusters on the production of collagen and elastin (Table VII). At the same time, the glycine-proline -lysine ratio and the valine-alanine ratio were kept constant to reduce the number of variables. In this study, the variable becomes the cluster of AAs characterizing the single protein. The compositions of the blends were as follows:

Blend E: Elastin AA Cluster: 29% of the total mixture
Blend D: Elastin AA Cluster: 39%
Blend G: Elastin AA Cluster: 44%
Blend F: Elastin AA Cluster: 49%

The results are summarized in Fig. 4 and show that in mixture E, with the lowest amount of elastin AA, there is no significant production of elastin itself, confirming the need for specific amounts of such AAs in order to produce this protein.

The above results show that the production of elastin is not linearly related to the quantities of the AAs thereof present in the mixtures. It therefore

Table V. *Relative quantification of collagen IV protein performed considering Negative Control (NC) at 120 h as calibrator sample.*

Sample	COLIV
NC	1
A	1.685
B	1.957
C	2.547*
D	2.278

***p<0.01 and *p<0.05 (Student t-test)*

Table VI. *Relative quantification of elastin protein performed considering Negative Control (NC) at 120 h as calibrator sample.*

Sample	ELN 55 kDa	TROPOELN 70 kDa
NC	1	1
A	0.805	1.82
B	4.105**	2.24*
C	2.403	1.65
D	1.999	2.16

***p<0.01 and *p<0.05 (Student t-test)*

Table VII. *Percentage of aminoacid composition of the tested formulations in Second phase.*

		Mixture D	Mixture E	Mixture F	Mixture G
COLLAGEN	Glycine	30.2	35.9	25.2	27.9
	L-Proline	22.7	27	18.9	20.9
	L-Lysine HCl	3.3	3.9	2.7	2
	L-Leucine	4.2	4.2	42	4.2
ELASTIN	L-Valine	16.8	16.7	28.2	25.3
	L-Alanine	22.8	12.3	20.8	18.7

***p<0.01 and *p<0.05 (Student t-test)*

Table VIII. *Summary of the effects of tested mixture of amino acids and hyaluronic acid on ELN and COLIV gene and protein expression in human fibroblasts, after 120 h of treatment.*

	Gene Expression		Protein	
	ELN	COLIV	ELN	COLIV
A	-	-	-	-
B	-	-	↑	↑
C	↑	↑	↑	↑
D	↑	↑	↑	↑

Up-regulation (↑) or no effect (-), compared to negative control (NC).

follows that increasing the concentration of elastin AAs results in an increased protein response up to a certain point. Indeed, 49% of elastin AAs have a non-optimal elastin production (towards control).

Collagen IV protein production is linearly connected to the amounts of the respective AAs present. Low collagen AA concentrations are directly related to low stimulation of collagen IV. It follows that further reducing the concentration of collagen AAs compared to those tested would certainly not be enough to stimulate collagen synthesis.

DISCUSSION

In addition to the important physical and chemical properties that make it a largely used molecule in clinical applications, HA has an interesting biological action on cell adhesion, proliferation and migration, cytokine release and the synthesis of extracellular matrix proteins in connective tissues. It has been shown that the biological action of HA can be synergistically enhanced by adding small active molecules such as AAs, thus increasing the possible application of this polymer in different fields of medicine and in the therapy or revitalization of connective tissue in particular.

The aim of this study was to assess the effects of different mixtures of AAs and HA on the production of extracellular matrix proteins, in particular: elastin and collagen IV, a major component of basal lamina.

For this purpose, a transcriptomic dynamic approach was applied to human primary dermal

fibroblast cultured in monolayer (from the breast skin of a 44-year-old donor, responsive to elastin synthesis), which was cultured for 5 days in the presence or absence (negative control) of products, while monitoring the expression kinetics of the selected genes after 24, 72 and 120 hours via qRT-PCR. The production of proteins was verified by Western blot analysis after 120 h of treatment.

Regarding the effects on transcriptional activity, as a general consideration, it can be noted that there was no change in gene expression until 72 h, possibly because the timing was insufficient for the dermal fibroblast monolayer to express and produce functional fibres.

In a recent study (14), it was demonstrated that 6.2% HA containing several active molecules, including AAs, progressively increased ELN expression in dermal fibroblasts, reaching a maximum at 72 hours.

This data supports the hypothesis that, in fibroblast monolayer, the expression of ECM proteins (in particular elastin) is not an immediate process. The presence of stimulation at 24-72 h is attributable to the different experimental design (different mixtures tested and application method)

In our model, an overexpression occurred after 120 h of growth with treatment and, as assumed, the test items exerted a differential action, on ELN and COLIV in particular. As summarized in Table VIII, compared to the negative control (untreated), formulation A, which can be considered an experimental benchmark [since its activity has been investigated in various studies (9-10,13)], it did not induce any up-regulation of the selected genes, although its ability to stimulate the transcription of collagens I and III has been previously reported (13). This formulation contains a combination of AAs, principally glycine and proline (present in large quantities in collagen molecules) and, following the assumption that the ECM protein synthesis can be enhanced by increasing the local bio-availability of specific constituent AAs, it can be argued that the AAs in formulation A are not the most effective for improving non-collagenous ECM proteins.

To verify this idea, other mixtures were formulated based on the AA composition of elastin (15), while decreasing the general glycine and proline content, introducing the specific hydrophobic residues, valine and alanine (in formulation B), doubling the amount of leucine (in formulation C) and increasing the amount of valine and alanine (in formulation D). Of the three products, C and D induced a significant up-regulation of ELN and COLIV genes. With regard to ELN, it seems that proline, alanine and valine are important for the biosynthesis thereof and this indication seems to be confirmed by the Avantaggiato and colleagues (14) study in which ELN gene expression was stimulated by a product that also contained AAs. Interestingly, collagen IV was also stimulated by mixtures C and D, thus indicating that those formulations are able to act on other components of the EMC matrix.

In order to verify the biological effects of the formulations on ECM component production, the ex-novo bio-synthesis of elastin and collagen IV was verified by Western blot analysis after 5 days of treatment. As reported in Table V, formulation A did not have any effect (as expected from RTqPCR data), while all the other mixtures (B, C and D) induced an increase in protein production. Interestingly, in connection to elastin, the products seemed to act differently on the two protein forms, insoluble α-elastin and soluble tropoelastin, thus indicating that different formulations can enhance the amount of the precursor (which can act as a reservoir for elastin recovery in the event of tissue damage) or promote protein maturation. Surprisingly, mixture B stimulated protein synthesis although no increase in gene expression was detected. This result could relate to the fact that differences between mRNA and protein levels can be observed depending on several factors, including the kinetics of transcription and translation phases, which can be influenced by local culture, cellular response to the stimulus, etc. (17).

The second phase of the study, which was conducted to clarify the interactions between the different clusters of AA, demonstrated that, given that the total amount of AAs in all the mixtures was always the same, a mixture containing AA amounts of both proteins must be selected in order to give a significant response and a significant production of both collagen and elastin. This also proves the

existence of a ratio between the 2 clusters (AA elastin/AA collagen) that guarantees an adequate and balanced response to fibroblast, collagen and elastin gene expression and production.

Moreover, the data of this pilot study must be confirmed and extended in order to allow in-depth investigation of the underlying mechanism of action.

This study, conducted on human dermal fibroblast monolayer using a transcriptomic dynamic approach, has shown the efficacy of different proprietary mixtures of AAs and HA in modulating the biosynthesis of extra cellular matrix proteins and in promoting elastogenesis in particular. It has been demonstrated that varying the quality and quantity of AAs in the mixtures allows elastin expression to be increased at gene and protein level by maintaining collagen stimulation, and of type IV collagen in particular.

This preliminary data can be a starting point for further studies on the biological activity of new mixtures of AAs and HA and on their future application in the medical field.

CONFLICT OF INTEREST: A. Orlandini has received consultancy fees from Professional Dietetics. The other Authors declare no conflicts of interest relevant to this article.

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