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Skin morphological and functional changes evaluation after amino acid replacement therapy

The modern stage of aesthetic medicine development consists of using safe certified products with well-known mechanism of action and proven clinical efficacy. That is why we observe the integration of scientific works aimed to the unbiased assessment of various cosmetic procedures outcomes and investigations of mechanisms that ensure this outcome into a routine clinical practice.

During our trial we examined patterns of structural and functional changes in the skin of patients from different age groups after a course of intradermal injections of medications containing a combination of various amino acids that have functionally importance for the collagen synthesis.

Skin structural proteins

Various mechanisms of skin aging are well-known at the present time. Most of them are stipulated by fibroblasts functional activity reduction: synthesis and rejuvenation of the extracellular matrix components (collagen, elastin, fibronectin, glycosaminoglycans, proteoglycans) are reduced with age, followed by matrix disorganization and delayed wound healing [1-6]. The most significant factor for skin appearance is disruption of skin main structural component homeostasis - collagen.

Synthetic potential of fibroblasts is hard to be overestimated: one activated mature cell is able to produce up to 3.5 million macromolecules of procollagen each day. [7] The part of mature collagen accounts for about 30% of the total protein content in the human body, and up to 70% of the skin proteins content. More than 20 types of collagen are known for the present time. Collagen types are determined by various tissues, depending on the role of given protein in the structure and function of the specific organ or tissue. Thus, collagens of the I and III types, which compose up to 90% of the skin dry basis, are organized into large fibers bundles, which form a 3D reticular structure. This reticulum determines biomechanical properties of the skin. Collagen of the IV type is the main part of the basal membrane, as well as blood vessels and skin appendages. Collagen of the VII type creates anchoring fibrils, and collagen of the VI type - permeates all skin layers like a reticulum [8-11].

Unique biomechanical properties of collagen fibers (flexibility and tensile strength coupled with rather low elasticity) are determined by primary and spatial structure of this protein.

Collagen molecules consist of three polypeptide α-chains. More than 20 types of α-chains have been identified. Most of them consist of more than 1 000 amino acids. Type I collagen molecule is a triple superspiral, which consists of one α2-spiral and two α1-spirals. All α-chains are differ by amino acid sequence, but the primary structure has several general trends [11, 12].

According to the results of recent studies, the structural feature of collagen α-chains is that 33.7% of all amino acids residuals are presented by glycine, 13.3% - by proline, 9.7% - by hydroxyproline, 11.6% - by alanine. The polypeptide chain of collagen consists mostly with triplets ‘gly-X-Y’, where ‘X’ and ‘Y’ could be represented by any amino acid. But, as a rule, the position ‘X’ is presented by proline, and the position ‘Y’ by hydroxyproline and hydroxylysine. Structural fragment ‘Gly-Pro-hydroxypropyl’ is called ‘collagen melody’ [11].

Collagen structure doesn’t contain cysteine and tryptophan. Such AA as histidine, methionine and tyrosine are presented in trace amounts.

Hydroxyproline is the next amino acid in the collagen. This amino acid is presented almost only in this protein. It is synthesized by hydroxylation of proline in embodied polymer chain, that is during post-translational modifications. This stage of collagen fibrils formation is most vulnerable to ascorbic acid deficiency.

Each of the dominant amino acids in the collagen chain is vitally important for high-grade collagen fibrils formation. Proline causes the formation of a polypeptide chain bends. These bends are crucial for stabilizing the left-twisted α-spiral chain. "Hard" amino acids - proline and hydroxyproline – reduce the rotation of the polypeptide rod, thereby increasing the stability of the triple spiral. Glycine molecule has a hydrogen atom instead of radical group. That is why glycine always locates at the intersection of α-chains, ensuring tightly fitting of all chains [12].

In contrast to strong collagen fibrils which are able to resist heavy loads, elastin possess rubber-like properties. Elastin filaments could be stretched much longer than their initial length, but after load elimination filament returns to the initial folded structure.

Elastin contains about 800 amino acid residues, which are presented mainly by non-polar amino acid radicals, such as glycine, valine and alanine. Elastin contains a lot of proline and lysine, and very small amounts of hydroxyproline, and hydroxylysine is completely absent.

Several amino acids - arginine, citrulline, glutamine, ornithine - stimulate tissue physiological regeneration and reparation. Glutathione, cysteine, methionine, histidine, arginine have antioxidant properties [10]. Hydroxyproline stimulates collagen synthesis in vitro and enhances the dermal origin cells proliferative activity (U.S. Patent 6692754).

The balance between collagen synthesis and degradation determines the integrity and functional completeness of tissues protein frame, including the dermis. This balance disruption leads to adverse consequences. In particular, the skin aging process is associated with collagen synthesis inhibition due to the synthetic activity of fibroblasts reduction.

In people of 80 years and older collagen synthesis decreased by 75% and degradation is increased by 75% compared with people of 18-29 years [3, 4]. As a result, there is an overall reduction of the collagen types I and III in dermis and collagen types I and III ratio decrease/ These processes are correlated with age [13]. Fragmented collagen is accumulated simultaneously: fragmented fibers are much more rigid and chaotic oriented [14]. This skin essential process is exacerbated by irreversible collagen structure modification due to new cross-links formation. These links are formed by simple sugars groups during enzymatic and non-enzymatic reactions, including free radical oxidation process. Glycation end products (AGEs) gradually accumulate in the intercellular matrix and change its properties significantly, resulting in skin appearance affection, rejuvenation ability reduction and blood vessels condition deterioration [15]. Just these changes are observed in diabetic patients.

Skin single exposure to UV emission reduces collagen production by 80% [16]. Chronic UV exposure increases the matrix metalloproteinases expression, which is followed by collagen degradation increase [2, 17].

Thus, collagen structure and content changes in the skin are observed both in natural aging and in photoaging, which leads to disruption of the skin frame and causes wrinkles formation. [18]

To enhance collagen synthesis in the skin the modern aesthetic medicine is encouraged to use various damaging effects (peeling, mesoroller therapy, fractional laser, radio-frequency exposure, ultrasound, etc.), as well as application of protein hydrolysis end products - amino acids - to the skin.

Amino acids role in the treatment of dermis age-related changes

Collagen synthesis and degradation processes are auto-regulated. Amino acids and peptides take active part in this regulation. Thus, the polycation poly-L-lysine inhibits the collagen biosynthesis and polyanion poly-L-glutamate stimulates this process [19]. Studies conducted in 1970 in USSR confirmed the hypothesis that the stimulating effect of the endogenous and exogenous collagen degradation products, including amino acids, on collagenesis in fibroblasts is performed by biofeedback mechanism [20].

The most active and physiological complex, which could activate the collagen types I and III synthesis and normalize collagens metabolic balance, is a functional cluster of amino acids, including glycine, proline, lysine and leucine. This cluster become the basis for various cosmetics products, dietary supplements, wound healing drugs for dermatology, combustiology and dentistry. Age-related skin changes modern treatment is called amino acid replacement therapy (AART) [21-23].

In cosmetics we use injectable products Jalupro and Jalupro HMW (Professional Dietetics, Italy). These products are registered in the Russian Federation as health care products and are intended for intradermal application (marketing authorization number 2011/102240). It is composed of amino acids mixture (glycine 50%, proline 37%, lysine 6%, leucine 7%) and sodium hyaluronate solution (1% in Jalupro and 2% in Jalupro HMW). Viscous solution of hyaluronic acid has anti-inflammatory action, and provides an extended release of amino acids into the tissues.

The solution for injection is prepared ex tempore by mixing the powder of amino acids and hyaluronic acid solution.

It has been shown that the active ingredients in AART drugs provide fibroblasts chemotaxis migration into the injected area, stimulate neocollagenesis, optimize the scarring process, improving skin quality, accelerate wound healing and reduce the recovery period after invasive procedures. Intradermal injection of Jalupro increases the skin thickness, improves its elasticity, smoothes the relief. All these actions are stipulated by collagen regeneration and accumulation [21-24].

Indications for AART procedure in aesthetic medicine are as follows: face and body skin photo- and physiological aging, stretches of different duration, premedication for invasive aesthetic interventions (plastic surgery, dermabrasion, laser treatment, high-frequency pulsed light therapy, radio wave therapy, ultrasound lift, micropunctural therapy and etc.) and long term rehabilitation.

Contraindications include following conditions: skin diseases in the treatment area, pregnancy, lactation, cancer and autoimmune diseases, hypersensitivity to the drug components.

The injection of Jalupro solution is conducted as a traditional mesotherapy method: the skin is treated by antiseptic solution and, if necessary, local anaesthesia is performed by anesthetic cream; the injection is performed into middle layers of the dermis - where the metabolic and proliferative active fibroblasts are located in large quantities.

Various injection methods could be used - linear, short-linear, papular etc. It is recommended to perform gentle massage of the treated area after injection in order to reach even distribution of the solution. This technique could be applied to treat facial skin, neck, decollate, arms, hands and other areas of concern.

Single dose (2.5 mL Jalupro HMW and 3.0 mL Jalupro) could treat two anatomic regions. Average volume of one dose is 1.25–1.5 mL.

Basic course include 4–6 procedures. Procedures are performed every 1-2 weeks. The visible results are reached after basic course performance twice a year. Supportive courses application is recommended every month.

AART clinical outcome could be described as skin tension turgor increase, micro-and macrorelief

alignment, skin color improvement, pigmentation aligning. However, any clinical observations are quite subjective. That is why clinical outcomes are the target for criticism of mesotherapy opponents.

In order to perform an objective evaluation of AART results we conduct a special clinical trial. It makes us possible to confirm clinical observations by objective instrumental images of the skin.

AART effectiveness assessment - clinical and instrumental trial

Materials and methods

We have observed 58 females aged 35-65 years with skin symptomatic involutional changes of various degree. Females with following conditions were excluded: skin diseases, autoimmune diseases, systemic blood diseases, exacerbation of chronic somatic disorders, pregnancy, intake of supplements or antioxidants additives and other biologically active compounds that could affect the quality of the skin within last 6 months.

All females have signed informed consent form to participate in the study. After that all participants were divided into two age groups: 35-45 years and 45-65 years (hormonal mechanism of skin aging is possible).

In order to correct the skin involutional changes the following treatment was performed: 4 procedures were conducted once per week. During each procedure Jalupro drug 1.5 ml was injected i.c. using micropapular technique (the distance between injections is 1-1.5 cm). Special attention was devoted to the periorbital area (total volume was not more than 0.5-0.6 ml), peri auricular area and the lower third part of the face.

Participants observation and instrumental diagnostic was conducted before the first procedure, 1 month and 4 months after treatment course completion.

The results were assessed by confocal laser scanning microscopy (MAVIG Vivascope 1500, Germany-USA). This method could visualize the collagen and elastin fibers architecture and the reticular dermis. Microrelief assessment was conducted by visual monitoring method (Visioscan VS 98, Courage + Khazaka electronic GmbH, Germany). Data obtained were processed by SELS software.

Epidermis and dermis structural patterns were evaluated by 2D ultrasound scanning (Dermascan C Ver. 3, Cortex Technology, Denmark) with a signal frequency of 20 MHz. Skin biomechanical properties were evaluated before and after AART using cutometry technique (Cutometer MPA 580, Courage + Khazaka electronic, Germany).

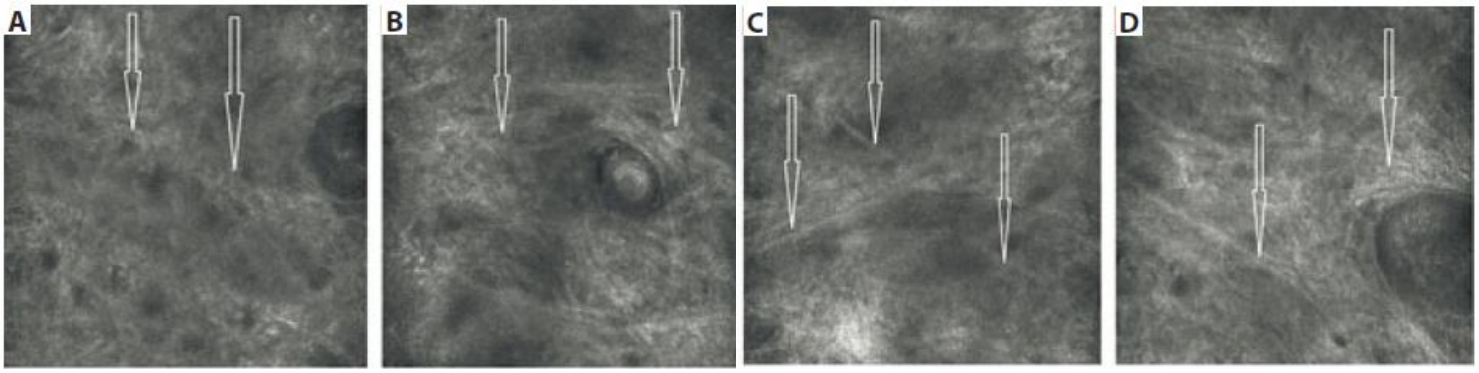
Data obtained were evaluated by parametric (t-test) and nonparametric (paired Wilcoxon test, Mann-Whitney test) methods. Statistical significance threshold was established at $p \leqslant 0.05$.

Results and discussion

Confocal laser scanning microscopy (KLSM) method revealed contrast areas in connective tissues in patients aged 45-65 years at baseline. Fragments of high laser reflection (particularly bright areas) and areas with low refraction (darker areas) were revealed among them. This is an indirect evidence of protein fibers reduction. The same females presented fiber disorganization and chaotic location. All of these changes were correlated with age to some extent. In the group of patients 35-45 years of symptoms of fiber disorganization were revealed only in isolated cases. Data obtained allowed to identify objective criteria for dermal involutional changes severity assessment.

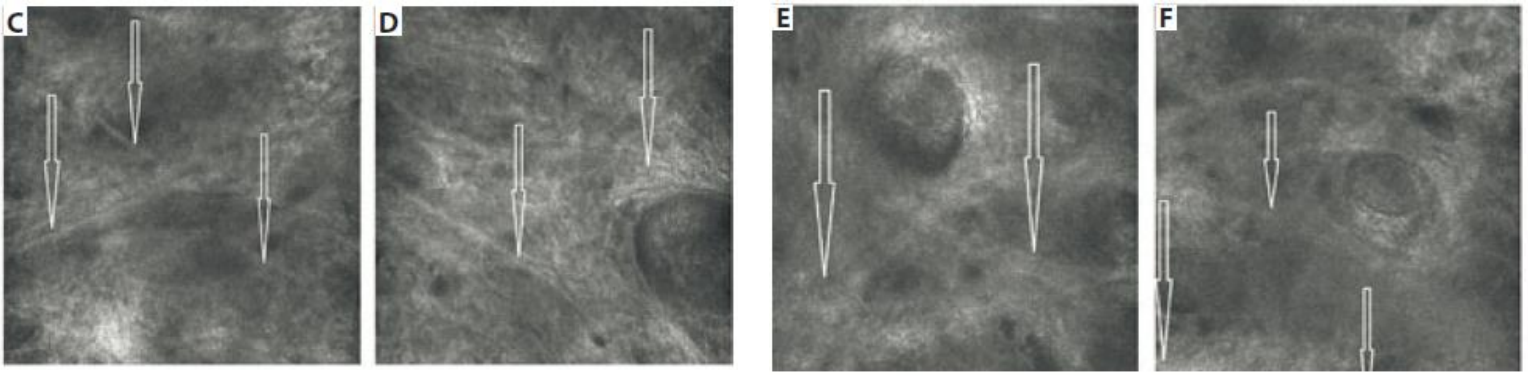
Repeated examination - 1 month after AART completion - in both age groups revealed significant changes. Thin fibers evenly distribution signs were observed in the papillary layer. These fibers formed a reticular structure. Signs of fiber quantitative improvement, low refraction areas reduction, sites of fibers disorganization reduction or disappearance (Fig. 2) were observed in dermis reticular layer. This pattern was maintained 3 months (or 4 months after AART completion): fibers better distribution, fibers thickening. This means the positive trend in dermal layer improvement.

A number of structural changes were identified during comparing the data of skin ultrasound scanning before and after the AART. Following changes were observed (Fig. 3):



Female N. 46 years old. A - Collagen fiber bundles in papillary and reticular layers are marked by arrows.
B - Arrows at the pictures before and after AART pointed the areas with fibers structure changes, fiber frame of the derma improvement.

Female I. 48 years old. C and D - Collagen fiber bundles in papillary and reticular layers are marked by arrows.



Female I. 48 years old. C and D - Collagen fiber bundles in papillary and reticular layers are marked by arrows.

Female T. 57 years old. E - Collagen fibers in papillary and reticular derma are marked by arrows. F - Areas with low refraction grade reduction (areas of derma structure disorganization) are marked by arrows.

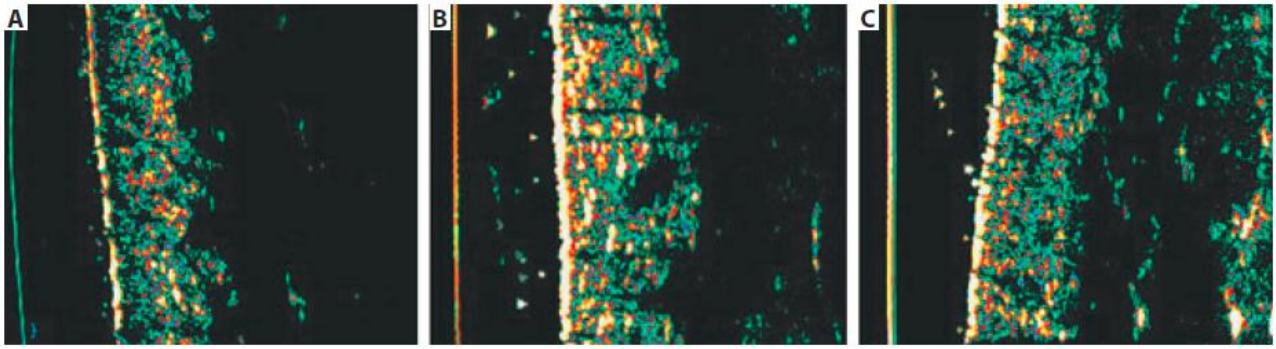


Figure 3. Skin structure change trend after AART (US scanning result)
Female O., 51 years old. A – before therapy; B – 1 month after therapy; C – 4 months after therapy

- epidermis and dermis thickening, epidermal layer induration and alignment;
- hypoehoic regions total area reduction by 8.45% ($p<0.05$);
- echosignal intensity increase by 13.2% ($p<0,05$), followed by more even echosignal distribution. This fact is indirect evidence of collagen fibers increase and dermal fiber frame reconstruction.

Analysis of the AART treatment results have shown, that we can talk about the dermis structure normalization, basic substance content increase and signs of fibrous frame deformation reduction. All changes were more pronounced in patients of older age group.

Ultrasound scanning has revealed that AART treatment in females of 45-65 years can adjust signs of dermis structural involutional disorganization. This changes were manifested as a significant skin thickening, turgor increased, wrinkles reduction. Signs of restructured dermis revealed by ultrasound scanning after AART persisted during 4 months later.

Elastometry data confirmed skin biomechanical parameters changes after therapy.

Total elasticity parameter R2 was increased comparing to basic level. This parameter reflects skin ability to return the original state after stretching:

- in the group 35–45 years – average increase was by 1.04% 1 month after therapy; and by 2.1% 4 months after therapy ($p<0.05$);
- in the group 45–65 years – average increase was by 4.2% ($p<0.05$) 1 month after therapy and this level was maintained during 4 months after therapy.

Differences from baseline and between groups were considered statistically significant. The clinical value of a sustained skin elasticity improvement is hard to be overestimated: this treatment offers effective prevention of wrinkles formation and gravitational ptosis degree.

Visual inspection has revealed different skin microrelief in patients of various age groups before and after i.c. injection of Jalupro drug.

Skin microrelief is determined mainly by two factors: epidermis water balance and dermis protein fibers condition [24]. Skin upper layers condition determines the smoothness and scabrities of the skin microrelief – the superficial parameters. And the quantity, quality and spatial orientation of the collagen and elastin fibers in the dermis determine fine wrinkles formation - microrelief [25].

Table 1

Skin relief changes after AART				
Parameter	35–45 years		45–65 years	
	Before therapy	1 month after therapy	Before therapy	1 month after therapy
Volume (microrelief), U.	51.13 ±1.34	48.63±2.10	68.43±3.10	50.31±0.15
Ser (scabrities), U	38.40±0.11	33.12±0.17	45.8±0.21	40.17±0.10
Sew (wrinkleness), U	2.30±0.10	2.08±0.30	2.64±0.52	2.41 ±1.10

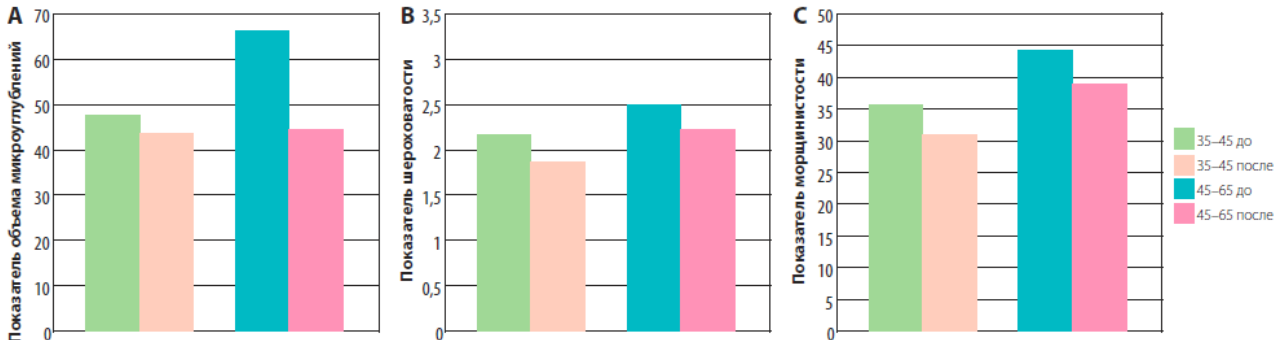


Figure 4. Parameters changes trend before and after AART: A – Microrelief; B – Scabrities; C – Wrinkleness

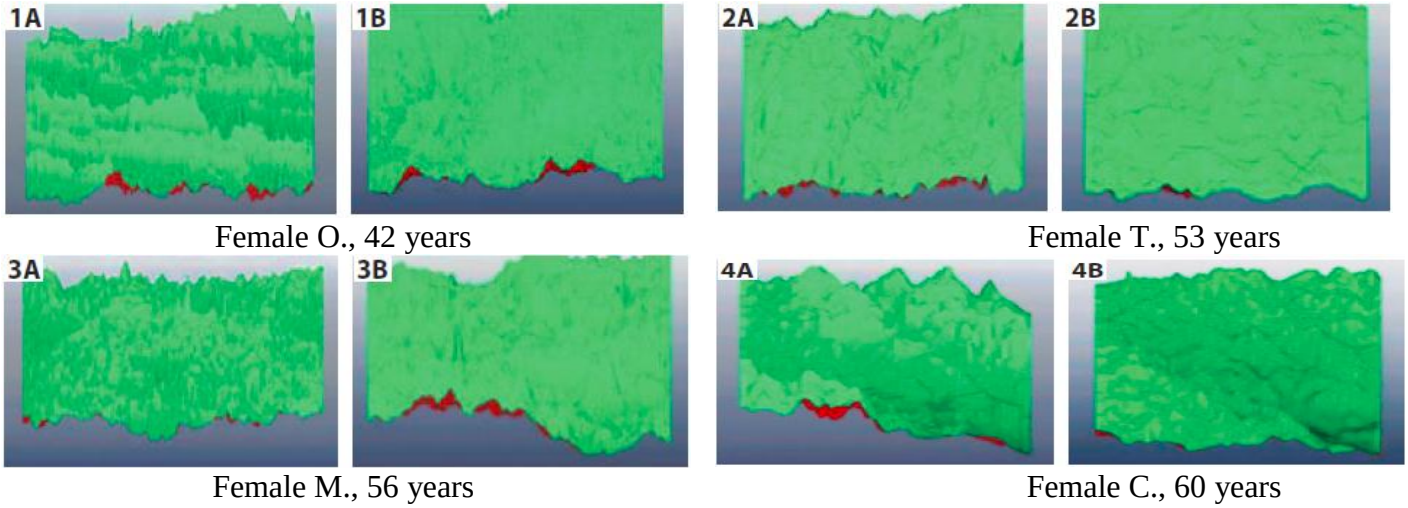


Figure 5. Skin surface 3D imaging (visioscanning) – periorbital area. Before (A) and after (B) Jalupro injections.

In assessing the skin microrelief before and after AART therapy the initially increased parameters were decreased: scabrities (Ser), wrinkleness (Sew) and microrelief rugosity (Volume) (Table 1, Fig. 4).

Presented visioscan images depict microrelief smoothing in females of both age groups after amino acid replacement therapy (Fig. 5).

Based on obtained results we could conclude that the administered amino acid replacement therapy, which included 4 i.c. injections of Jalupro drug, reduced the rate of wrinkling, scabriting and improved skin microrelief. That changes result in skin clinically improvement: skin color and luster improvement.

Resumed data are presented in Table 2.

Instrumental diagnostic results of the skin before and after AART course using Jalupro drug			
Age group	Before therapy	1 month after therapy	4 month after therapy
35–45 years	Confocal laser scanning microscopy (CLSM)		
	fibers minimal changes; single areas of fibers disorganization in single participants	quantitative and qualitative improvement of fibers; areas of fibers disorganization reduction or disappearance	Result persists without changes
	Ultrasound scanning		
	areas of uneven echosignal distribution; areas of epidermis uneven structure	epidermis and dermis thickening; echosignal intensity increase; epidermis structures alignment	Result persists without changes
	Cutometry		
	–	Total elasticity parameter increase by 1.04% from baseline	Total elasticity parameter increase by 2.1% from baseline
	Visual investigation		
	Microrelief rugosity volume parameter 51.13±1.34 SEr-scabrities 2.3±0.1 to 2.08±0.3 SEw wrinkleness 38.4±0.11	Microrelief volume decrease by 5.0%; scabrities decrease by 13.8%; wrinkleness decrease by 9.6%	Data are in process
45–65 years	Confocal laser scanning microscopy (CLSM)		
	A large number of fibers disorganization areas; chaotic fibers location; lack of spatial orientation	fibers disorganization areas number decrease; fibers even location; reticular structure formation.	Areas of low refraction grade significant reduction
	Ultrasound scanning		
	epidermis and dermis thickness reduction; thinning and irregular structure of the epidermis; echosignal uneven distribution	epidermis and dermis thickness increase; echosignal even distribution in dermis, echosignal intensity increase by 13.2 %	Result persists without changes
	Cutometry		
	–	Total elasticity parameter increase by 4.2 % from baseline	Data preserved at the same level
	Visual investigation		
	Microrelief rugosity volume parameter from 68.43±3.1 SEr-scabrities 2.64±0.52 SEw-wrinkleness 45.8±0.21	Microrelief rugosity volume parameter 50.31±0.15; SEr-scabrities 2.41±1.1; SEw-wrinkleness 40.17	Data are in process

Conclusion

The experience of the amino acid replacement therapy with Jalupro drug for aesthetic indications is more than 6 years. Research data and analysis of the European and Russian clinical experience have shown, that this drug has the ability to stimulate regeneration and anti-inflammatory action. Mesotherapy course using Jalupro drug in combination with TCA chemical peels not only improve the performance and prolong the effect of the peels, but also significantly reduce the severity and duration of postpeeling erythema [20-23]. Combination therapy improves outcomes of fractional photothermolysis [11]. However, the main criterion in results of mesotherapy assessing, including combination with other treatment, is based on clinical outcome. Clinical and instrumental study performance allowed us not only to give a reasonable response to skeptics, but also to clarify which effects could be achieved during the intradermal injection of the amino acids complex.

Skin instrumental examination by confocal laser scanning microscopy and 2D ultrasound scanning revealed the skin structure improvement after Jalupro injections. The most evident changes were achieved in patients of older age group (45-65 years). Data obtained by cutomentry showed

sustained improvement of skin biomechanical properties, particularly skin elasticity increase. Optical visual monitoring revealed prominent positive changes of the skin microrelief – the key factor that determines the clinical picture of "rejuvenation", that we were seeing after the treatment. Thus we've got objective evidence of the possibility to skin restructuring after intradermal injection of the functionally significant cluster of amino acids, that previously we have observed on the basis of clinical observations.

In our opinion, the amino acid replacement therapy with the Jalupro drug could be recommended to use in clinical practice for skin involutional changes prevention and treatment, especially in elderly patients. Younger patients are recommended to administer this therapy to prevent the skin premature aging after photodamage. Recommendation of the amino acid mesotherapy application to improve and prolong the results of various stimulating treatments (i.e., chemical peels, laser therapy, fractional photothermolysis, phototherapy (IPL), dermabrasion, radiofrequency and ultrasound lift) and to prepare the skin for plastic surgery is still relevant. Combination of mesotherapy with injection contouring therapy or botulinotherapy could significantly improve the overall aesthetic outcome mainly due to the skin quality improvement.

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