LETTER TO THE EDITOR

PEGYLATED HYALURONIC ACID FILLER ENRICHED WITH CALCIUM HYDROXYAPATITE TREATMENT OF HUMAN SKIN: COLLAGEN RENEWAL DEMONSTRATED THROUGH MORPHOMETRIC COMPUTERIZED ANALYSIS

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To the Editor,

Functional fibroblast collapse is the most important factor of the extracellular matrix modifications of the connective tissue of aged skin (1). The progressive decreasing of the natural process of synthesis and renewal of extracellular matrix components, such as collagen and hyaluronic acid of the ground substance, constitutes the main cause of the loss of support, elasticity and hydration of the skin in older individuals, with the consequent appearance of lines and wrinkles. The use of implant materials such as gel-based on cross-linked hyaluronic acid were introduced and used with promising success. Due to the appreciated filling properties and immunological compatibility, hyaluronic acid-based fillers in dermatology and aesthetic medicine are today widely used for the restoration of lost volumes and the correction of wrinkles.

Thanks to technological achievements, a PEGylated HA-CaHA filler (Stimulate®, MatexLab SA, Lugano, Switzerland) has been recently introduced as a biocompatible, injectable filler for facial soft-tissue augmentation and wrinkle correction, with well described crosslinking properties (2) and characterized by interesting rheological and filling properties, which seems to offer considerable advantages in terms of biosafety and collagen stimulation (3).

In mammalian tissues, collagen molecules are ordered in a parallel orientation, providing a natural birefringence. This optical property is enhanced by Picrosirius red dye, a selective histochemical stain for collagen, due to a dye molecule alignment parallel to the axis of each collagen molecule constituting elementary collagen microfibrils. When viewed under polarized light, Picrosirius red stained collagenic structures change in colour depending on the size, type and the three-dimensional organization

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of collagen molecules (4-6).

The current study used Picrosirius red in combination with circularly polarized light microscopy to examine the effects of PEGylated HA-CaHA filler treatment on the molecular organization of collagen, which is closely related to a possible stimulation of fibroblasts. In our histological preparations, the observed colour due to the birefringence is depending by the type of collagen, permitting the identification of newly formed collagen (4, 6).

MATERIALS AND METHODS

Five women scheduled for redundant abdominal skin removal received subdermal injection of a small volume (1 ml) of PEGylated HA-CaHA 1% filler (NEAUVIA Stimulate®) 2 months before surgery using a 25-gauge, 1-inch sharp needle and controlled slow withdrawal of the syringe. At the time of surgery, two specimens were obtained from the surgically excised skin, one 1 cm apart from the injection site, and another from an untreated zone at a distance of 10 cm to serve as a control. The study was conducted in accordance with the ethical principles that had their origin in the Declaration of Helsinki and all subjects provided written informed consent.

Biopsy specimens were fixed by direct immersion in a 4% paraformaldehyde/phosphate buffer solution for 24 h and then processed for light microscopy by dehydration, embedded in paraffin, and sectioned. The 5-µm thick sections were stained with hematoxylin and eosin (Merck, Darmstadt, Germany) to facilitate localization of the injected areas in the skin. Adjacent sections, with the same 5-µm thickness for all samples (control and treated), were stained with Picrosirius red dye solution 0.1% (Sirius red F3B, Sigma-Aldrich, St Louis, MO, USA) in a saturated aqueous solution of picric acid for 1 h, in the same staining solution and for the same length of time. To identify mature and newly-formed collagen, and therefore highlight possible regeneration/renewal of the connective tissue, a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) equipped with suitable circularly polarizing (in the condensor stage of the microscope) and analyzer (in the microscope tube above the objective lens) filters, was used. The filters were aligned so that the background in the field of view was as dark as possible. Morphometric computerized analysis of biopsy specimens from PEGylated HA-CaHA-treated and control tissues was performed using ImageJ software version 1.49h (Waine Rasband, National Institutes of

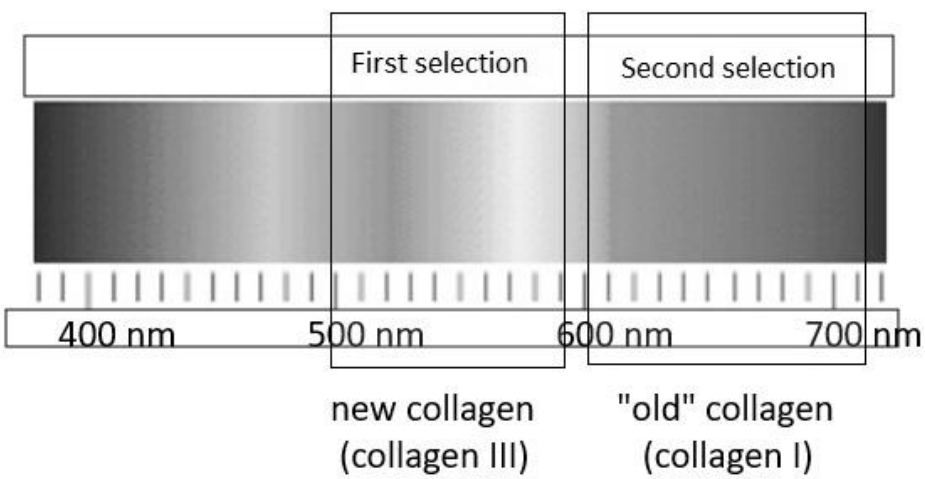

Fig. 1. *Two bands selected for segmentation of images as Fig. 2a (control) and Fig. 2d (following PEGylated HA-CaHA treatment). The second selection identifies the mature, stabilized, 'old' collagen fibers, the first one identifies the newly-formed fibers following stimulation of fibroblasts by PEGylated HA-CaHA treatment.*

Health, Bethesda, USA) on microscopic digital images recorded with a Nikon DS-Fi2 high definition 5-megapixel color CCD camera head (Nikon, Tokyo, Japan).

RESULTS

The original images of tissue sections acquired by circularly polarized light microscopy from untreated (control) and PEGylated HA-CaHA filler-treated skin (two months after injection) are respectively illustrated in Fig. 2, a and d. Pixel signals from both control and treated areas, which were extracted from the images and underwent morphometric analysis, permitted to provide a quantitative evaluation of the newly-formed collagen, representative of the regenerating effects of HA-CaHA filler treatment. Following the HA-CaHA filler treatment, the proportion of thin fibers formed by newly-formed collagen (type III) increased significantly ($p<0.01$) relative to the proportion of the thick mature old collagen (type I) fibers. In contrast, the collagen content of the control tissue consisted almost exclusively of thick mature collagen type I fibers. The comparative results are presented in Fig. 3.

DISCUSSION

Our results complement the findings from previous studies using HA-CaHA-based fillers (7-11) and demonstrate that PEGylated HA-CaHA significantly stimulates the formation of new collagen type III fibers. The association of Picrosirius red staining with

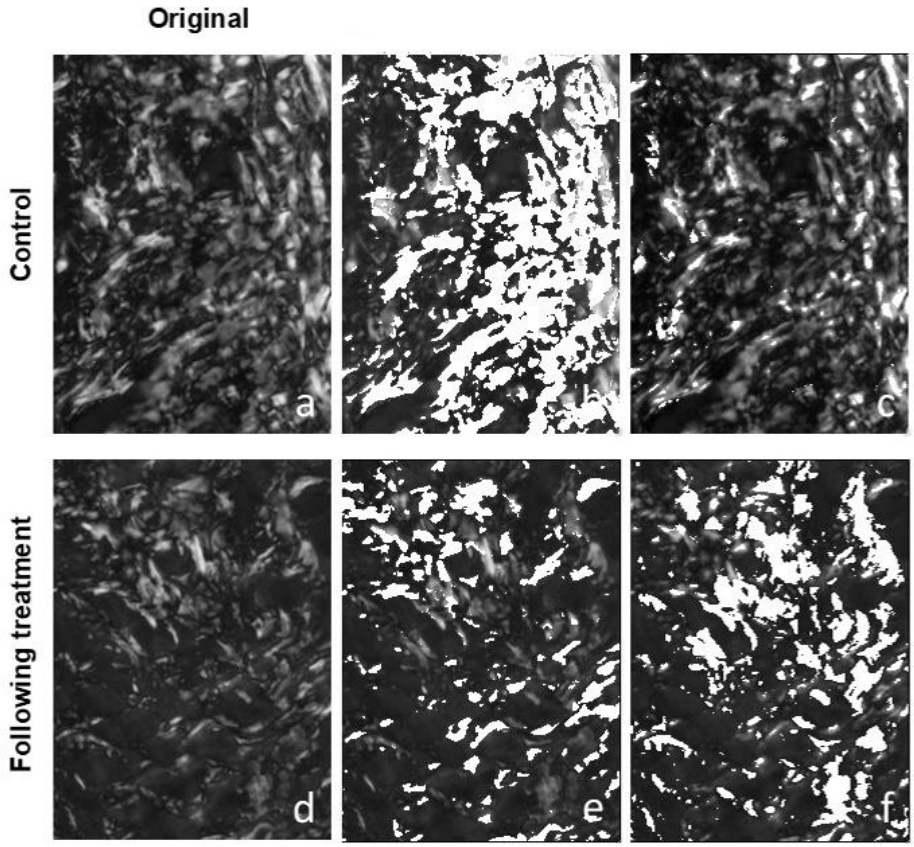

Fig. 2. *Histological subdermal sections stained with picrosirius red, observed under the microscope equipped with circularly polarized light.* ***a****) Control.* ***d****) Two months following pegylated HA-caha injection. Computerized image analysis was performed for both a) control and d) pegylated HA-caha-treated areas using imagej software (NIH).* ***a*** *and* ***d****) original photomicrographs of histological sections stained with Picrosirius red.* ***b*** *and* ***e****): segmentation of images a and d;* ***c*** *and* ***f****) segmentation of images a and d.*

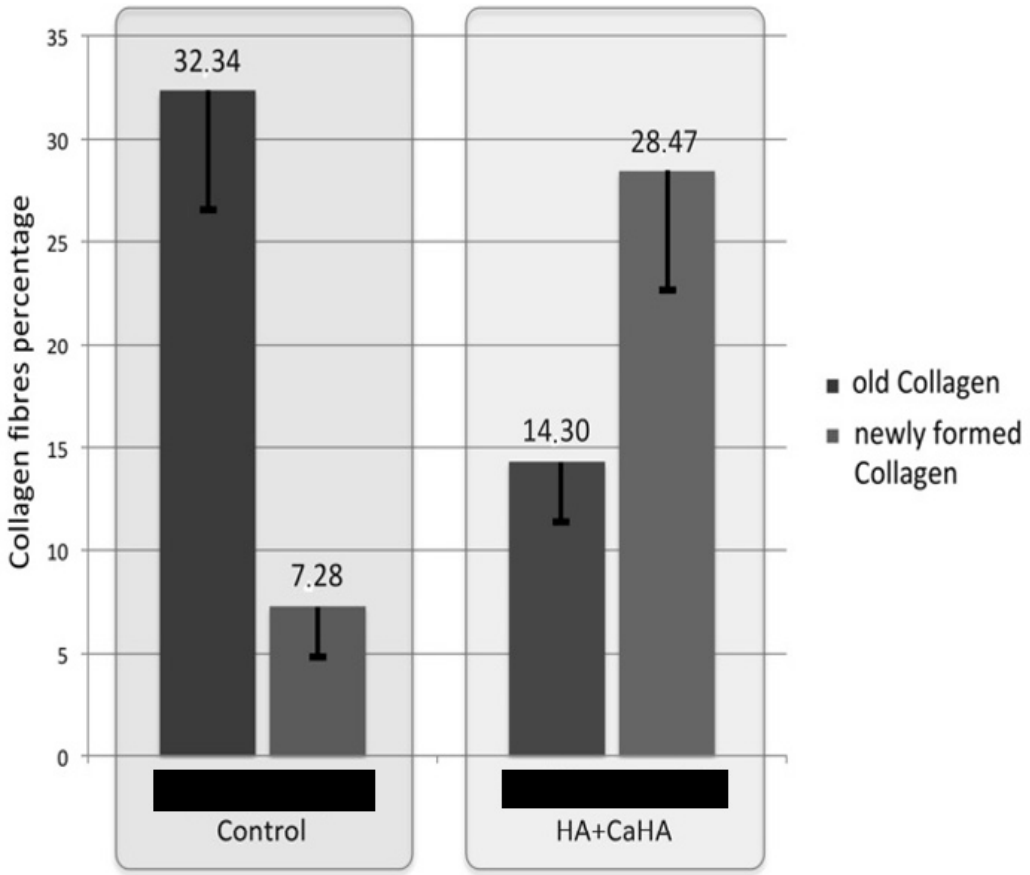

Fig. 3. *Morphometric evaluations of the regenerative effects of PEGylated HA-CaHA treatment. The statistically significant ($p<0.01$) increase in newly-formed collagen (type III) following treatment (lighter coloured bar) in comparison with the mature collagen type I (darker bar) is the result of a specific stimulation of fibroblasts to synthesize new collagen fibers for an effective renewal of the subdermal connective tissue.*

circular polarized light microscopy represents a useful method for the identification and characterization of collagen in human skin. This study demonstrated significant differences in collagen content between control skin and skin treated with subdermal injection of PEGylated HA-CaHA (26 mg/ml) + CaHA (1%) filler. It was possible to evaluate the content of mature collagen type I and the content of newly formed collagen type III. Differently from control samples, image analysis and computerized morphometry on samples from sites of PEGylated HA-CaHA filler injection demonstrated a predominance of birefringent fibres. It would now be of interest to use this sensitive method of visualizing new collagen to conduct further studies at longer time intervals between injection of PEGylated HA-CaHA and abdominoplasty surgery with biopsy withdrawal.

REFERENCES

1. Fisher GJ, Varani J, Voorhees JJ. Looking older: fibroblast collapse and therapeutic implications. Arch Dermatol 2008; 144:666-72.
2. Monticelli D, Martina V, Mocchi R, Rauso R,

Zerbinati U, Cipolla G, Zerbinati N. Chemical characterization of hydrogels crosslinked with polyethylene glycol for soft tissue augmentation. Maced J Med Sci 2019; 7(7):1077-81.

3. Hassan W, Dong Y, Wang W. Encapsulation and 3D culture of human adipose-derived stem cells in an in-situ crosslinked hybrid hydrogel composed of PEG-based hyperbranched copolymer and hyaluronic acid. Stem Cell Res Ther 2013; 4:32-43.
4. Zerbinati N, Lotti T, Monticelli D, et al. In vitro evaluation of the biosafety of hyaluronic acid PEG cross-linked with micromolecules of calcium hydroxyapatite in low concentration. J Med Sci 2018; 6(1):15-19.
5. Zerbinati N, Rauso R, Gonzalez P, et al. In vitro evaluation of collagen production on human fibroblasts treated with hyaluronic acid peg cross-linked with micromolecules of calcium hydroxyapatite in low concentration. J Biol Regul Homeost Agents 2017; 31(2 Suppl 2):87-90.
6. Cheng W, Yan-Hua R, Fang-Gang N, Guo-An Z. The content and ratio of type I and III collagen in skin differ with age and injury. Afr J Biotechnol 2011; 10:2524-29.
7. Yutskovskaya Y, Kogan E, Leshunov E. A randomized, split-face, histomorphologic study comparing a volumetric calcium hydroxylapatite and a hyaluronic acid-based dermal filler. J Drugs Dermatol 2014; 13:1047-52.
8. Van Loghem J, Yutskovskaya YA, Werschler WP. Calcium hydroxylapatite: over a decade of clinical experience. J Clin Aesthet Dermatol 2015; 8:38-49.
9. Courderot-Masuyer C, Robin S, Tauzin H, Humbert P. Evaluation of lifting and antiwrinkle effects of calcium hydroxylapatite filler. In vitro quantification of contractile forces of human wrinkle and normal aged fibroblasts treated with calcium hydroxylapatite. J Cosmet Dermatol 2016; 15:260-68.
10. Coleman KM, Voigts R, DeVore DP, Termin P, Coleman WP 3rd. Neocollagenesis after injection of calcium hydroxylapatite composition in a canine model. Dermatol Surg 2008; 34(Suppl 1):S53-55.
11. Berlin AL, Hussain M, Goldberg DJ. Calcium hydroxylapatite filler for facial rejuvenation: a histologic and immunohistochemical analysis. Dermatol Surg 2008; 34(Suppl 1):S64-S67.