# Comparative Clinical and Histomorphologic Evaluation of the Effectiveness of Combined Use of Calcium Hydroxyapatite and Hyaluronic Acid Fillers for Aesthetic Indications

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**KEYWORDS**

• Dermal fillers • Hyaluronic acid • Calcium hydroxylapatite • Immunohistochemistry

**KEY POINTS**

- Fillers, such as hyaluronic acid and calcium hydroxylapatite, can be used to both volumize and stimulate the body's own collagen to a certain degree.
- Combination approach using multiple modalities in specific sequence for the safe and effective treatment of the aging face.
- Simultaneous injection of the studied products is possible from the point of view of safety, but different levels of administration will be more optimal, and provide a more pronounced remodeling effect on the skin.

## INTRODUCTION

The age-related changes that we observe on the faces of our patients in the form of wrinkles, folds, creases, changes in the contour of the face, and loss of volume are associated with changes that occur at the levels of all 5 anatomic layers of the face: bone skeleton, ligaments, muscles, adipose tissue, and skin. The work of an aesthetic doctor should consist of the creation of an optimal comprehensive protocol of procedures that are aimed at those problems with a solution that will lead to maximum results in the form of clinical visual improvement, as well as having a therapeutic effect on the soft tissues of the face. In order to achieve naturalness and harmony, it is necessary to smooth out moderately pronounced and deep facial wrinkles and folds, treat the hypertonus of facial muscles, replenish lost volumes, moisturize the skin, and restore its elasticity.

It is important to understand that to achieve such a complex task of facial rejuvenation, combination treatment of technology and soft tissue augmentation with injectable fillers and neuromodulators must be used. The question is when and how to combine such aesthetic interventions

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safely and effectively on the face, hands, neck, and decollete.

Hyaluronic acid and calcium hydroxylapatite can help in replenishing lost volume as well as stimulating the dermis to produce new collagen, respectively. Individually these materials have been used for treating facial lipoatrophy, associated with either human immunodeficiency virus or age, or more generally facial soft tissue volume loss.[1–5] The materials have also been approved to create a smoother and/or fuller appearance in the face, including nasolabial folds, cheeks, and lips and for increasing the volume of the back of the hand.[6,7]

Hyaluronic acid has been studied, either for facial treatments or for treatment of atrophic scars, and proved its effectiveness and has a favorable and well-characterized safety profile.[1,2,8] It has become the gold standard that is compared with all dermal filler studies. Calcium hydroxylapatite is the only biodegradable filler that immediately restores lost volume and simultaneously stimulates the production of natural skin collagen to achieve long-term results.[9] It is a versatile injectable implant and a valuable tool for short- and long-term cosmetic and reconstructive treatments. This injectable is often used in conjunction with botulinum toxin as well as other injectables and energy-based devices. Its effectiveness and safety have been also demonstrated in several studies.[10–12]

Combined aesthetic interventions have widespread application in clinical practice, but results are infrequently reported at scientific meetings and in the medical literature, as there are many variables that need to be investigated. Given the complexity of facial aging, expert consensus supports a combination approach using multiple modalities in specific sequence for the safe and effective treatment of the aging face.[13,14]

The aim of this study is to evaluate histomorphologic findings following Belotero Volume (CPM-HA V) (Merz Aesthetics, Raleigh, NC, USA), a hyaluronic acid–based volumizing filler, and compatibility of a combination of CPM-HA V and Radiesse (Merz Aesthetics, Raleigh, NC, USA), a gel consistent of calcium hydroxylapatite microspheres in carboxymethylcellulose vehicle, in 2 patient groups.

## MATERIAL AND METHODS

### *Study Design*

This is an open-label, prospective, pilot, randomized, comparative clinical study and immunohistochemical analysis in healthy female volunteers, 35 to 45 years of age with body mass index (BMI) less than 21. Each subject signed the informed consent form to participate in the study. Ethical approval for the study was obtained in accordance with the ethical principles of the declaration of Helsinki and the International Conference on Harmonisation of Good clinical practice December 2019 to July 2020.

### *Study Participants*

In total, 8 female volunteers aged 35 to 45 years with BMI less than 21, who had indications for lower face, neck, and décolleté lifting, participated in the study. All study subjects completed the study.

During the study, the study subjects had one intradermal CPM-HA V injection into integumentary tissues of the periauricular area (subdermal injection) and followed simultaneous subdermal Radiesse injection into the same area.

Eight study subjects met inclusion/exclusion criteria and were randomly assigned to experimental groups with a 1:1 allocation as per a computer-generated randomization schedule into 2 groups: Group I (CPM-HA V injection and followed Radiesse injection in 1 month)—4 subjects and Group II (CPM-HA V injection and simultaneous Radiesse injection)—4 subjects. Subjects were followed-up for 5 months.

Each subject had a case report form that included information on the date and frequency of procedures, gender, age, area of product injection, biopsy area marking, procedure tolerability assessment score, and assessment of any side effects.

### *Study Treatments*

The study was composed of 3 visits.

Volunteers in Group 1 had intradermal CPM-HA V injections into integumentary tissues of the periauricular area (subdermal injection) at stage 1 (D01). The 27 G 19 mm needle is inserted at an angle of 35° to 40° into the dermis. Standard Radiesse was injected intradermally into one's integumentary tissues of the periauricular area (subdermal injection) in a month at stage 2 (M01). The 27 G 19 mm needle is inserted at an angle of 35 to 40° into the dermis.

Volunteers in Group 2 had intradermal CPM-HA V injections into integumentary tissues of the periauricular area (subdermal injection). Then standard Radiesse was injected intradermally into one's integumentary tissues of the periauricular area (subdermal injection). Both injections were performed at stage 1 (D01). The 27 G 19 mm needle is inserted at an angle of 35° to 40° into the dermis.

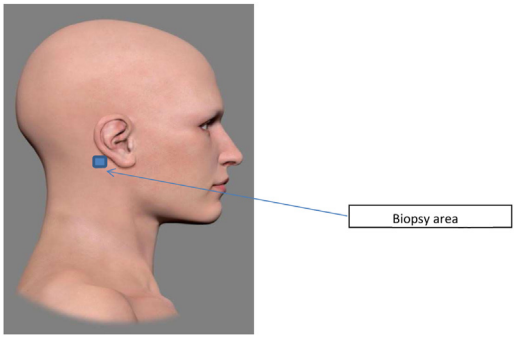


**Fig. 1.** Punch biopsy area scheme.

Punch biopsy from the treated periauricular area was performed at all 3 visits (**Fig. 1**).

## *Study Endpoints*

### *Efficacy*

As an endpoint the histomorphologic evaluation based on the type I collagen, the type III collagen, and the elastic fibers' analysis using immunohistochemistry test (IHC) in terms of the compatibility of the combination of CPM-HA V and Radiesse was assessed at stage 2 (M01) and at stage 3 (M06) compared with the baseline data.

For the IHC test the patient biopsies samples were fixed in 10% neutral formalin and paraffin embedded. Series of paraffin sections, 4 μm each, were prepared and stained using hematoxylin and eosin, Van Gieson, and Weigert elastic stain. ICH reactions were carried out by antigen retrieval in a retriever according to the standard protocol. Monoclonal anticollagen I antibodies (murine-derived monoclonal antibodies produced by Santa Cruz [sc293182], clone 3G3, dilution 1:100), collagen III (murine-derived monoclonal antibodies produced by Santa Cruz [sc-166316], clone B-4, dilution 1:100), and vascular endothelial growth factor (VEGF) (rabbit polyclonal antibodies produced by Abcam [ab-183100], dilution 1:100) were used. Reactions were performed with positive and negative controls in the absence of primary antibodies.

A comparative analysis of type I and type III collagen, elastin, and other histomorphologic characteristics (presence of an inflammatory reaction, angiogenesis) included 3 zones of the sample (subepithelial, superficial, and deep dermal layers). Staining intensity (on a point-based scale) of samples for histology and immunohistochemistry was assessed using a semiquantitative method. Mild staining corresponded to marker expression of 2 points: moderate—4 points and high—6 points.

### *Safety*

Adverse events were assessed and recorded at each of the 3 visits.

## *Statistical Analysis*

The study was planned to be a pilot, and sample size was not determined. Descriptive statistics are given for each studied parameter. Mann-Whitney test was used to analyze the between-group differences for the study efficacy endpoints. Within the group comparison of the study efficacy endpoints was conducted using Wilcoxon test.

The statistical analysis was performed with the Stata application software (StataCorp, USA) version 14.

# RESULTS

## *Study Population*

Eight screened healthy female volunteers met the inclusion/exclusion criteria and were randomized 1:1 into 2 groups. All study subjects completed

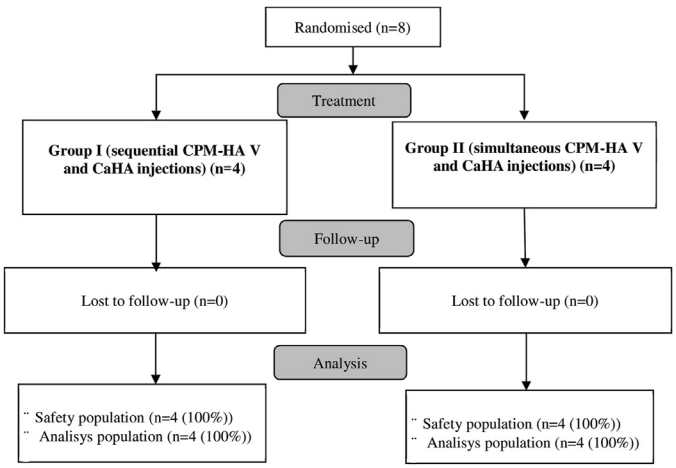


**Fig. 2.** Patients' distribution.

the study without significant protocol deviations. Safety population includes all the patients who received at least one dose of study medications (**Fig. 2**).

All the enrolled subjects were divided into 2 groups:

- Group 1 to 4 patients who received CPM-HA V injection and followed injection of standard Radiesse in a month.
- Group 2 to 4 patients who received CPM-HA V injection and simultaneous injection of standard Radiesse.

Available demographic and clinical data show no difference between the 2 groups before treatment; the 2 groups are comparable in terms of the tested parameters.

## *Efficacy Outcomes*

### *Histomorphological evaluation of the efficacy*

**Comparative histology and histochemistry changes** Comparative analysis of skin biopsy histomorphology before, 1 month and 5 months after the combined injections of CPM-HA V and Radiesse were performed. Eight punch skin biopsy specimens from the retroauricular area of all 8 study subjects were analyzed.

At the baseline in both groups elastic stain showed no elastic fibers in the subepithelial and superficial layers of the dermis; instead, these were observed as individual fibers within the perivascular and periglandular tissues in the deep dermal layers (0 points).

One month after the single injection of CPM-HA V, Group 1 revealed remodeling of the epidermis due to the accumulation of elastic fibers and an increase in the amount of extracellular matrix. There are no pathologic changes in the epidermis; the layer did not show any thickening, and there was preserved stratification. Sample evaluation showed no signs of hyperkeratosis. Both papillary and reticular layers of the dermis were not thick and were relatively easy to distinguish. There were multidirectional collagen fibers with a moderate number of slightly unevenly distributed fibroblasts and fibrocytes. The dermis (with hematoxylin and eosin staining and Van Gieson staining) showed extracellular matrix accumulation with the deposition of collagen bundles. There were slightly more vessels (small veins, venules, capillaries, and arterioles) in the dermis compared with stage 1 ($p<0.05$) that revealed lumen enlargement and relatively even distribution of the sample, and their number is similar to those seen at stage 1. Elastic stain revealed elastic fibers (2–4 points) mainly in the superficial and deep dermal layers located around vessels and skin appendages ($p<0.05$).

In Group 2, there was mild remodeling of the dermis due to extracellular matrix accumulation, signs of angiogenesis, and the accumulation of elastic fibers. With hematoxylin and eosin staining and Van Gieson staining, the dermis also revealed deposition of collagen bundles in the extracellular matrix. There were few lymphocytes present in an uneven distribution in the field of view and were typically located in perivascular spaces within the walls of small blood vessels as well as close to some hair follicles. They were not indicative of an inflammatory reaction at the site of filler implantation. There were slightly more vessels (small veins, venules, capillaries, and arterioles) present in the dermis compared with stage 1($p<0.05$). These findings may also be considered morphologic signs of

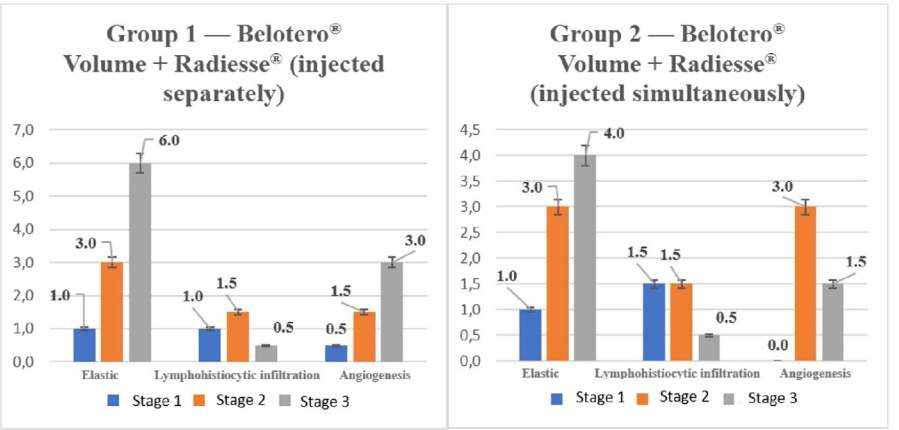


**Fig. 3.** Comparative analysis of skin biopsy histomorphology before, 1 month and 5 months after the combined injections of CPM-HA V and Radiesse.

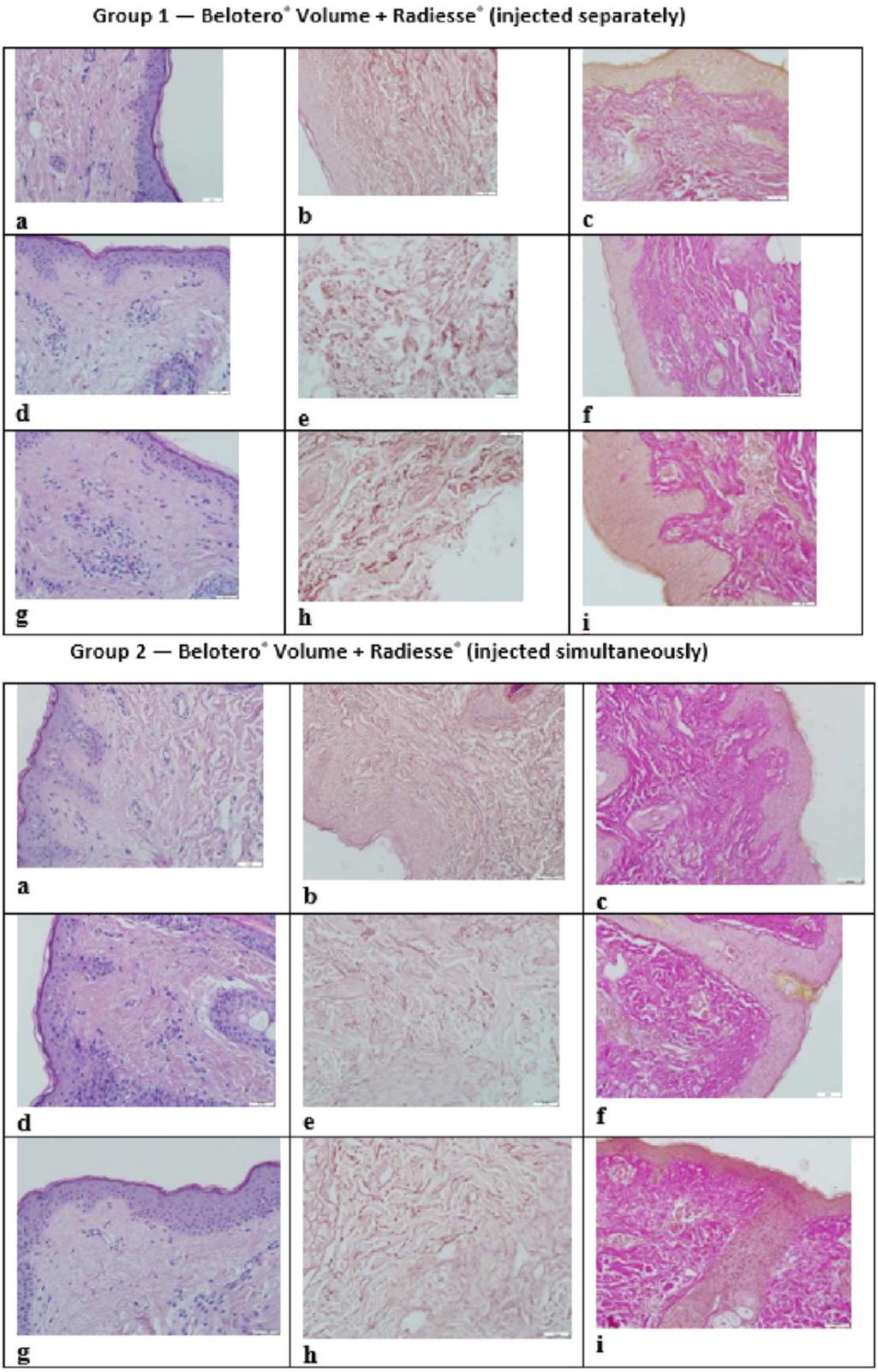


**Fig. 4.** Microscopic photographs. Histologic and histochemical characteristics of skin biopsies before treatment (*A*, *B*, *C*), 1 month (*D*, *E*, *F*), and 5 months after the combined injections of CPM-HA V and Radiesse (*G*, *H*, *I*). Stains: hematoxylin and eosin (*A*, *D*, *H*), orcein (*B*, *E*, *H*), and Van Gieson picrofuchsin (*C*, *F*, *I*), X 400.

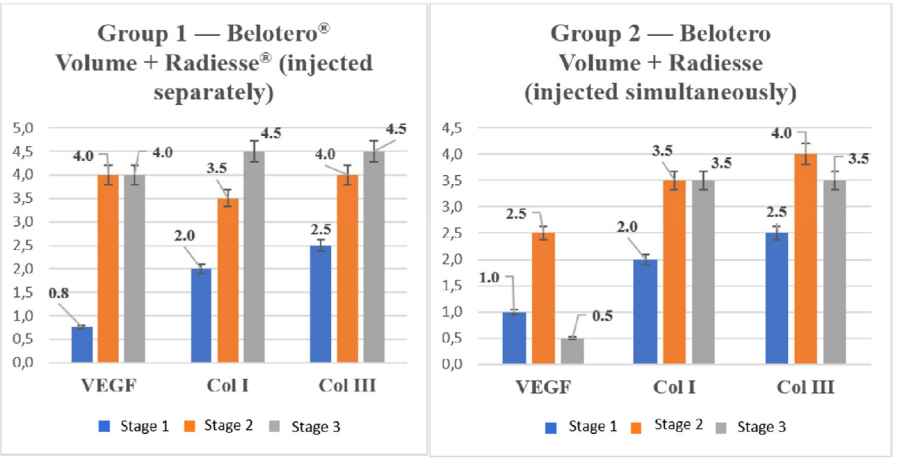


**Fig. 5.** Comparative analysis of skin biopsy histochemistry before, 1 month, and 5 months after the simultaneous injection of the combination of CPM-HA V and CaHA.

active connective tissue remodeling at the site of dermal filler injection (2–4 points). Elastic stain revealed elastic fibers ($P$<0.5) mainly in the superficial and deep dermal layers located around vessels and skin appendages (2–4 points).

A greater rate of angiogenesis (p<0.05) was observed in Group 2, 1 month after the simultaneous injection of CPM-HA V and Radiesse.

Five months after the combined injections of CPM-HA V and Radiesse comparative analysis of skin histomorphology revealed that both products caused remodeling in one's skin, whereas separate injection of CPM-HA V and Radiesse resulted in greater skin remodeling characterized by the following: trend toward ECM accumulation; more pronounced accumulation of elastic fibers (p<0.05); enhanced angiogenesis within 4 months after the injection (p<0.05).

The results are shown in **Figs. 3** and **4**.

**Comparative immunohistochemistry changes** In both groups at stage 1 before products injection type I collagen is observed as thin interlacing fibers in the dermis found mainly in the subepithelial and superficial layers. Type III collagen is observed as thicker interlacing fibers of similar location in the dermis and in an amount similar to that of type I collagen: collagen I/III ratio = 1.0. VEGF is observed as cytoplasmic staining in isolated vascular endothelial cells (0–2 points).

A comparative analysis of immunohistochemical characteristics of the tissues following the sequential injections of CPM-HA V and Radiesse as well as simultaneous injections of CPM-HA V and Radiesse showed that these interventions had a remodeling effect on the dermis although the separate injections of CPM-HA V and Radiesse had a relatively greater effect. This conclusion is supported by more intense type I to III collagen accumulation (p<0.5) in the dermis and preservation of the 1:1 ratio as well as intense angiogenesis (p<0.5) that provides sustained blood supply to the skin after sequential injections of CPM-HA V and Radiesse performed in Group 1.

The results are shown in **Figs. 5** and **6**.

### *Safety Outcomes*

During the study, no adverse events were reported.

## DISCUSSION

The results of this study demonstrate that the injections of the combined injections of CPM-HA V and CaHA lead to histomorphological improvement. A comparative analysis of histomorphological changes in tissues revealed that the products cause remodeling of the skin, with the combined use of CPM-HA V and standard CaHA having a considerable impact on aging and skin remodeling due to ECM accumulation of elastic fibers ($P$<0,5).

Previous studies reported an increase in collagen type III with a gradual equalizing of the ratio toward collagen type I.[11,15] This study, however, showed an increase in the amount of both collagen type I and collagen type III with the same ratio.

According to our results, the sequential injections of CPM-HA V and CaHA had a relatively greater remodeling effect on one's skin compared with the simultaneous injections of CPM-HA V and CaHA. This is confirmed by more intense type I to III collagen accumulation (p<0.5) in the dermis and preservation of the 1:1 ratio as well as intense angiogenesis (p<0.5) that provides sustained

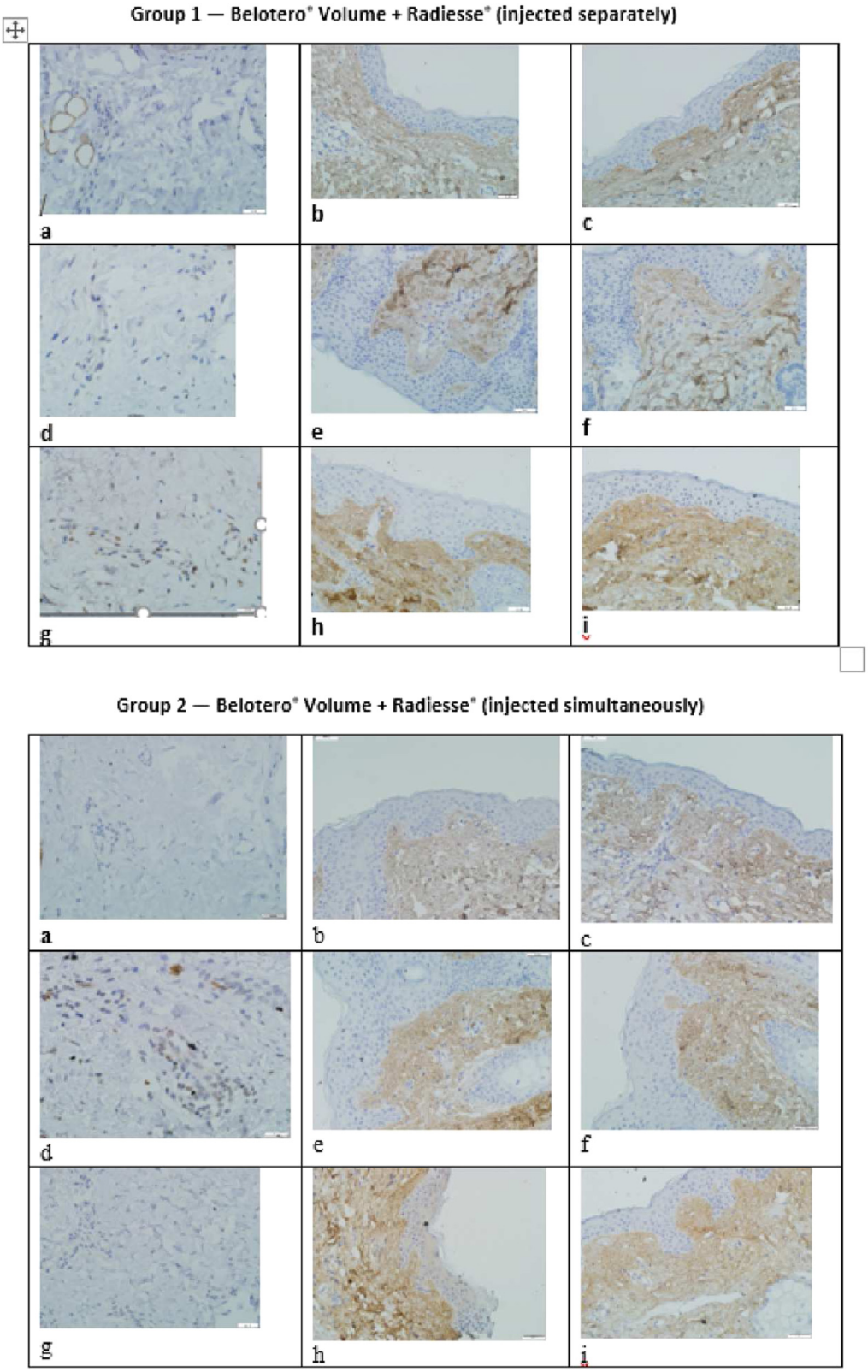


**Fig. 6.** Microscopic photographs. Immunohistochemical characteristics of skin biopsies before treatment (*A*, *B*, *C*), 1 month (*D*, *E*, *F*), and 5 months after the combined injections of CPM-HA V and CaHA (*G*, *H*, *I*). Immunoperoxidase reactions for VEGF (*A*, *D*, *H*), type I collagen (*B*, *E*, *H*), and type III collagen (*C*, *F*, *I*), Ч 400.

blood supply to the skin after consecutive injections of CPM-HA V and CaHA.

## SUMMARY

In conclusion, the data from this randomized, open, prospective, pilot, clinical study correspond to displaying the efficacy in terms of the histomorphological evaluation of the sequential injections of CPM-HA V and CaHA and simultaneous injections of CPM-HA V and CaHA. A comparative analysis of histology and immunohistochemistry of samples following the combined injection of CPM-HA V and CaHA revealed that phased injections of CPM-HA V and CaHA had a greater expected remodeling effect on one's skin compared with simultaneous injection of CPM-HA V and CaHA; this is confirmed by intense elastic fiber formation ($p<0.05$), type I to III collagen accumulation in the dermis ($p<0.05$) with the preserved 1:1 ratio, and intense angiogenesis ($p<0.5$) that provides sustained blood supply to the tissues. There is also evidence that simultaneous injection of CPM-HA V and CaHA at the same level is safe, confirming that there are no histologic and immunohistologic signs of inflammation and disturbance of tissue trophicity.

If we interpret the data obtained regarding clinical practice, we can draw the following conclusion: simultaneous injection of the studied products is possible from the point of view of safety, but different levels of administration will be more optimal, which will provide a more pronounced remodeling effect on the skin.

## OTHER INFORMATION

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## CLINICS CARE POINTS

- Hyaluronic acid and calcium hydroxylapatite may be combined in the same treatment to provide and added benefit.
- Care should be utilized when using calcium hydroxylapatite as, unlike hyaluronic acid, may not dissolve with hyaluronidase.
- Combination therapy is key to tackle the different signs of ageing.

## DISCLOSURE

The authors have no conflicts of interest when writing this author.

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