In vivo Bio-Integration of Three Hyaluronic Acid Fillers in Human Skin: A Histological Study

Christian Tran\textsuperscript{a} Pierre Carraux\textsuperscript{a} Patrick Micheels\textsuperscript{b} Gürkan Kaya\textsuperscript{c} Denis Salomon\textsuperscript{a}

\textsuperscript{a}Faculté de Médecine, Université de Genève, \textsuperscript{b}Private practice and \textsuperscript{c}Department of Dermatology, Hôpital Cantonal Universitaire, Geneva, Switzerland

Key Words
Hyaluronic acid · Hyaluronic acid fillers · Aesthetic medicine · Safety · Histology · Bio-integration · Esthélis\textsuperscript{®} Basic · Restylane\textsuperscript{®} · Juvéderm\textsuperscript{TM} Ultra

Abstract
Background: Hyaluronic acid (HA) formulations are used for aesthetic applications. Different cross-linking technologies result in HA dermal fillers with specific characteristic visco-elastic properties. Objective: Bio-integration of three CE-marked HA dermal fillers, a cohesive (monophasic) polydensified, a cohesive (monophasic) monodensified and a non-cohesive (biphasic) filler, was analysed with a follow-up of 114 days after injection. Our aim was to study the tolerability and inflammatory response of these fillers, their patterns of distribution in the dermis, and influence on tissue integrity. Methods: Three HA formulations were injected intradermally into the iliac crest region in 15 subjects. Tissue samples were analysed after 8 and 114 days by histology and immunohistochemistry, and visualized using optical and transmission electron microscopy. Results: Histological results demonstrated that the tested HA fillers showed specific characteristic bio-integration patterns in the reticular dermis. Observations under the optical and electron microscopes revealed morphological conservation of cutaneous structures. Immunohistochemical results confirmed absence of inflammation, immune response and granuloma. Conclusion: The three tested dermal fillers show an excellent tolerability and preservation of the dermal cells and matrix components. Their tissue integration was dependent on their visco-elastic properties. The cohesive polydensified filler showed the most homogeneous integration with an optimal spreading within the reticular dermis, which is achieved by filling even the smallest spaces between collagen bundles and elastin fibrils, while preserving the structural integrity of the latter. Absence of adverse reactions confirms safety of the tested HA dermal fillers.

Introduction

Hyaluronic acid (HA) is a natural, repetitive disaccharide macromolecule that exhibits no species or organ specificity in its natural form. It is composed of D-glucuronic acid and N-acetyl-D-glycosamine monosaccharide [1]. An average human body contains a total of 15 g of HA [2], half of which (7–8 g) is located in the skin [1]. HA half-life in the body can vary, but is less than 24 h in...
the skin [1, 3]. In its natural form HA is not immunogenic [4], and it has been used in different medical domains for 3 decades without exhibiting major adverse reactions [5]. Excellent tolerance and hygroscopic properties of HA render this molecule an ideal candidate to create volume in soft tissue such as skin [6]. HA fillers are therefore widely used for dermal enhancement in aesthetic medicine worldwide [5].

To overcome the limited lifetime of HA caused by its rapid enzymatic degradation in vivo, a cross-linking process that produces intermolecular bonds between HA macromolecules has been discovered in the sixties [7]. It stabilizes the superstructure of this otherwise linear macromolecule. At present HA fillers are derived from biotechnological processes utilizing streptococcus biofermentation and are generally cross-linked with a binding agent, 1,4-butaneediol diglycidyl ether [8]. Cross-linked fillers can be classified in two types: cohesive (monophasic) or non-cohesive (biphasic) depending on the technological processes used [8–12]. Cohesive (monophasic) fillers are composed of a single phase of HA, which is cross-linked once (monodensified) or continuously (polydensified) [8, 9]. These technologies and the resulting degree and type of cross-linking determine the viscoelastic properties of HA fillers. When injected intradermally, the effect of soft tissue volume and shape enhancement is in theory limited to 6–18 months depending on the type of filler used, the anatomical site and the individual patient’s metabolism [2, 10, 11]. Isolated reports of longer-lasting persistence of an HA filler in the dermis have been published [10]. In this study we selected three kinds of CE-marked HA fillers: a cohesive (monophasic) polydensified, a cohesive (monophasic) monodensified and a non-cohesive (biphasic) filler. All three products are widely used for various aesthetic indications and have a good safety record [3, 13–16].

A cohesive polydensified filler is manufactured with Cohesive Polydensified Matrix (CPM®) technology and is characterized by variable densities of cross-linked HA zones. This ensures optimal spreading of the gel into surrounding tissue allowing larger spaces to be filled with denser parts of the gel and finer pericellular tissue spaces to retain low-density gel [12, 16].

A cohesive monodensified filler is manufactured with Hylacross™ technology and is characterized as a highly cross-linked smooth gel as the result of homogenization of the manufacturing process [14].

A non-cohesive filler is manufactured with Non-Animal Stabilized Hyaluronic Acid (NASHA™) technology and is characterized as cross-linked particles suspended in a non-cross-linked HA matrix [13]. For a comprehensive review of different types of HA fillers, refer to Romagnoli and Belmontesi [3].

Verpaele and Strand [13] and Bogdan Allemann and Baumann [14] describe technologies and clinical experiences with non-cohesive and cohesive monodensified fillers, respectively. Prager et al. [15] compare the safety of these fillers to that of a cohesive polydensified filler in a controlled randomized clinical study. Flynn et al. [17] analyse the histological behaviour of the same fillers after 14 days. In our study we examined how three HA fillers with different visco-elastic properties behave as fillers in human dermis with a follow-up of 114 days after injection. Our aim was to study the tolerability and inflammatory response of these fillers, patterns of distribution in the dermis, and influence on tissue integrity. More precisely, we investigated the processes governing this tolerability at the cellular level, such as inflammatory response and granuloma formation, and correlated them with safety at the macroscopic level.

Materials and Methods

HA Fillers

Three HA fillers of different cross-linking processes were selected: (1) a cohesive (monophasic) polydensified filler of non-animal origin, manufactured using patented CPM technology – Estheris® Basic (Anteis SA, Geneva, Switzerland) also commercialized under Belotero® Balance in the USA by Merz Aesthetics Inc. (San Mateo, Calif., USA); Belotero Balance was approved by the FDA in November 2011; (2) a cohesive (monophasic) monodensified filler of non-animal origin, manufactured using proprietary Hylacross technology – Juvederm® Ultra (Allergan Irvine, Calif., USA) was approved by the FDA in 2006; (3) a non-cohesive (biphasic) filler of non-animal origin, manufactured using the patented NASHA technology – Restylane® (Q-MED, Uppsala, Sweden) was approved by the FDA in 2003. All three HA fillers selected for our study are CE marked and commercialized in Europe.

Subject Population

The study took place in the Department of Dermatology at the Hôpital Cantonal Universitaire de Genève, Switzerland. It was approved by the Hospital Ethics Committee and conducted in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki. Subjects of either sex, over 30 years old and able to provide written informed consent were included in the study. Exclusion criteria were any serious concomitant disease or uncontrollable medical condition requiring surgical and pharmacological treatments, known allergy to the local anaesthetic or medical device as well as participation in concurrent clinical investigations. Women who were pregnant or not using appropriate methods of contraception were also excluded from the study. All 15 subjects (13 women and 2 men, age range 41–65 years; mean age 52 years) enrolled in this study signed a written informed consent form.
Study Design and Procedures
Subjects attended 4 study visits: screening (days –14 to –1), baseline (day 0) when filler injections were performed, and follow-up visits on days 8 and 114 when biopsies were taken. The skin surface of the iliac crest region was thoroughly disinfected with a 2% alcoholic solution of chlorhexidine prior to any intervention. Injections and biopsies were performed by the same experienced physician in order to minimize any possible bias.

A volume of 0.2 ml of Esthélis® Basic (n = 15), and control isotonic saline (n = 15) were injected intradermally with a 30-gauge needle. Additionally, 10 subjects received an injection of Restylane®, and 5 subjects received an injection of Juvéderm™ Ultra. Three injections were performed on each side of the iliac crest region with a precise mapping and a polaroid picture (size 1/1) in order to be able to identify the injection sites.

Each injected product formed a papule of approximately 8–10 mm in diameter under the skin surface. On days 8 (n = 15) and 114 (n = 14), 3 biopsies (each filler and control) were performed on each side of the iliac crest. The area around the papules was locally anaeasthetized with lidocaine without epinephrine in a ‘ring’ fashion to avoid disturbance of the injection site. Four-millimetre punch biopsies were performed. The lesions were then stitched by the physician. The biopsy on day 8 was taken to observe the presence or absence of an early inflammatory reaction as well as the postinjection filler distribution. The biopsy on day 114 was taken to follow a possible chronic inflammatory response and to observe the state of degradation of the filler.

Histology
Skin samples were fixed in 10% phosphate-buffered formaldehyde, embedded in paraffin, and processed for histological analysis. Sections were cut at a thickness of 5 μm, mounted onto slides, and stained with haematoxylin-eosin, van Gieson elastin, toluidine blue, Alcian blue and colloidal iron, according to standard procedures.

Immunostaining
Paraffin-embedded sections were mounted onto slides, de-waxed in xylene, rehydrated in a graded ethanol series, and prepared for immunoperoxidase staining according to standard procedures. Primary antibodies included anti-CD3 (1:100; Dako Denmark A/S, Glostrup, Denmark), anti-CD20 (1:20; Dako Denmark A/S, anti-CD68 (1:750; Dako Denmark A/S), anti-α-actin (1:50; Dako Denmark A/S) and anti-procollen I (1:100; Biosystems Switzerland AG, Nunningen, Switzerland). After staining with the primary reagent for 1 h at room temperature, sections were washed, incubated with biotinylated affinity-purified secondary antibody for 30 min at room temperature, washed, and treated with avidin-biotin-peroxidase for 30 min at room temperature. The sections were then washed in buffered saline and incubated in 0.05% 3,3′-diaminobenzidine (Sigma Aldrich, Switzerland) and 0.03% H2O2 in phosphate-buffered saline at room temperature. All sections were examined under a microscope (Axiopt, Carl Zeiss Microscopy GmbH, Germany) using appropriate filters.

Transmission Electron Microscopy
Samples were fixed in 3% glutaraldehyde buffered in 0.1 M sodium phosphate, pH 7.4, embedded in epoxy resin (Araldit; Serva, Heidelberg, Germany), cut with a diamond knife and contrasted with uranyl acetate in methanol and lead citrate according to standard procedures. Image acquisition was performed with a Philips CM 10 Transmission Electron Microscope (Eindhoven, The Netherlands).

Results

Injection
Following injections of Esthélis® Basic, Juvéderm™ Ultra, Restylane® and isotonic saline solution, a papule formation was observed. Injection of the control isotonic saline solution formed a hemispheric homogeneous structure under the skin. Cohesive (monophasic) and non-cohesive (biphasic) HA fillers formed less symmetrical and homogeneous papules. Certain factors, such as dermal resistance of a given patient or position of the needle during injection, can impact the resulting shape. After 8 days papules corresponding to the fillers remained visible. If the operator pressed on the skin close to the punch biopsy, a small quantity of HA filler could be expressed through the pores. Extrusion of HA gel traces has also been observed after 114 days. At the isotonic saline injection site the skin appeared normal.

Histology
Haematoxylin-eosin stained cutaneous structures in pink (fig. 1). It can be seen that the skin structure is intact: no epidermal or dermal alterations or inflammatory infiltrates were detected, either 8 or 114 days after the injection. Furthermore, when toluidine blue staining was applied, only very few mast cells were observed in skin sections of tested fillers and control.

Alcian blue and colloidal iron stainings were used to detect dermal HA (fig. 1). Scant blue coloration in negative control (fig. 1a, e) shows the endogenous level of HA. For all tested HA fillers the faint blue coloration is similar in the papillary dermis, suggesting a lack of penetration of the gels into this area. In the reticular dermis remarkable differences are revealed between cohesive polydensified (fig. 1b, f), cohesive monodensified (fig. 1c, g) and non-cohesive (fig. 1d, h) fillers.

The cohesive polydensified filler Esthélis® Basic is distributed very similarly to endogenous HA in the control slide after 8 days (fig. 1b). The gel spreads centrifugally from the point of injection and reaches the papillary dermis layer without penetrating it. In this manner, distribution of this gel is most homogeneous compared to other tested fillers at both follow-up visits.

The cohesive monodensified filler Juvéderm™ Ultra forms small aggregates, which are less homogeneously distributed after 8 days (fig. 1c). The gel does not spread.
towards the papillary dermis as far as the cohesive polydensified filler does.

The non-cohesive filler Restylane® forms large aggregates distributed irregularly throughout the entire cross-section of the reticular dermis after 8 days (fig. 1d). The spreading of the gel is more restricted in the vertical axis, and thus it does not reach the proximity of the papillary dermis. These observations are corroborated by similar observations made by other groups [17, 18] who also give no indication of the fillers reaching the papillary dermis.

For all tested fillers we observed a decrease in the intensity of blue staining after 114 days (fig. 1f, g, h) in comparison to 8 days (fig. 1b, c, d). We hypothesize that this may be correlated with a reduced quantity of exogenous HA, although it cannot be excluded that it may be due to a reduced ability of HA to absorb the stains. The distribution patterns of the gel within the cross-section of the dermis after 114 days remained similar for each product as it was after 8 days.

In order to visualize collagen bundles and elastin fibres, histological sections were stained with van Gieson elastin solution, colouring collagen in red and elastin in black (fig. 2). It is assumed that white areas correspond to the presence of HA.

The cohesive polydensified filler Esthéli Basic is distributed very similarly to endogenous HA in the control slide after 8 days (fig. 2a, b). Furthermore, its distribution pattern is most homogeneous compared to other tested fillers. White spaces are uniformly spread among structural fibres stained in red and black, filling the narrowest spaces (fig. 2b, f).

The cohesive monodensified filler Juvéderm™ Ultra staining pattern shows small aggregates, which are less homogeneously distributed (fig. 2c, g). White spaces are more pronounced compared to figure 2b with respect to structural fibres stained in red and black.

The non-cohesive filler Restylane® staining pattern shows large aggregates pushing structural fibres stained in red and black (fig. 2d, h).

After 114 days the distribution pattern of the three HA fillers is the same as after 8 days in all studied samples. There is no change in characteristics of collagen and elastin viewed under high magnification (fig. 2f–h).

All histological results and observations are summarized in table 1.

**Immunolabelling**

Immunolabelling techniques have been used to characterize possible presence of an inflammatory infiltrate, a change in fibroblast population from quiescent fibroblasts to myocontractile fibroblasts or production of procollagen I. To this end, antibodies (table 1) have
been used to identify T and B lymphocytes, macrophages, myofibroblasts and procollagen I (table 1). No increase in the T or B lymphocyte population has been observed at the injection sites on days 8 or 114. Neither induction of myofibroblasts nor presence of procollagen I have been observed after immunolabelling with appropriate antibodies after 8 or 114 days following injection.

No granuloma has been detected on dermal sections 8 or 114 days following injection of the three HA fillers. Nevertheless, when compared to the control, a few isolated macrophages randomly distributed in the dermis have been observed in each of the samples. The results are summarized in table 2.

**Table 1.** Histological results and observations

<table>
<thead>
<tr>
<th>Staining</th>
<th>Labelled structure</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin-eosin</td>
<td>Cutaneous structures</td>
<td>Morphological conservation of epidermal and dermal structures</td>
</tr>
<tr>
<td>Colloidal iron, Alcian blue</td>
<td>Mucopolysaccharides/HA</td>
<td>Pronounced differences in distribution of non-cohesive, cohesive monodensified and cohesive polydensified HA fillers</td>
</tr>
<tr>
<td>Van Gieson elastin</td>
<td>Collagen and elastin fibres</td>
<td>Morphological conservation of collagen and elastin fibres</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Mastocytes</td>
<td>No increase in mastocytes</td>
</tr>
</tbody>
</table>

**Fig. 2.** a–h Staining with van Gieson elastin solution on days 8 and 114. Scale bars = 100 μm (a–d) or 40 μm (e–h).

**Transmission Electron Microscopy**

Fibroblasts, collagen fibrils and elastin fibres were visualized using transmission electron microscopy (TEM). No structural alterations were observed on cellular or extracellular matrix components (fig. 3). The cytosol of the fibroblasts was rich in rough endoplasmic reticulum and cytosolic vesicles, suggesting active protein synthesis. Additionally, we observed the presence of homogeneously distributed electron-dense zones. These zones correspond to areas which were occupied by the dermal fillers. Following dehydration and embedding procedures during sample preparation, these zones probably reveal the endogenous glycoprotein network of the extracellular matrix.
Discussion

As patients look towards less invasive and non-permanent methods for correction of rhytids, injectable dermal fillers based on cross-linked sodium hyaluronate are fast becoming one of the most frequently performed aesthetic procedures worldwide [5]. Considering the high demand for soft tissue augmentation procedures and a vast range of HA fillers that exist on the market, safety remains a paramount requirement for all products. It is thus particularly important to study bio-integration and tolerability of HA fillers after their injection into the skin. While safety has been demonstrated in numerous clinical studies [3, 13–16], cellular behaviour of HA fillers remains to be fully explored. To this end, we deliberately chose to inject the fillers in relatively large quantities into the immunogenic environment of the dermis, specifically selecting a non-mobile zone of the body. This experimental situation allowed us to adequately characterize HA fillers in terms of their tolerability and distribution in the dermis after 8 and 114 days.

Haematoxylin-eosin staining revealed that cutaneous structures remained morphologically intact and no inflammation was induced by the injection of all tested fillers after 8 and 114 days (fig. 1). Toluidine blue staining showed no increase in mastocytes in comparison to the negative control, suggesting absence of allergic or wound-healing response. Good tolerability of HA fillers was further supported by immunohistochemistry results, which revealed that none of the tested implants induced the occurrence of myofibroblasts in the injected dermis. Furthermore, we did not observe any increase in procollagen I, suggesting that dermal fibroblasts did not change their metabolic behaviour. Presence of only a few macrophages

Table 2. Immunolabelling results

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Labelled structures</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α-actin</td>
<td>Myofibroblasts/smooth muscle</td>
<td>Absence of myofibroblasts</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>T lymphocytes</td>
<td>No T lymphocyte infiltration</td>
</tr>
<tr>
<td>Anti-CD20</td>
<td>B lymphocytes</td>
<td>No B lymphocyte infiltration</td>
</tr>
<tr>
<td>Anti-CD68</td>
<td>Histocytes/macrophages</td>
<td>No granuloma formation; a few isolated macrophages in sections corresponding to the three HA fillers as opposed to control</td>
</tr>
<tr>
<td>Anti-procollagen I</td>
<td>Active fibroblasts</td>
<td>No increase in fibroblast activity around injected fillers</td>
</tr>
</tbody>
</table>

CD = Cluster of differentiation.

Fig. 3. a, b TEM micrographs of sagittal skin sections injected with: a cohesive polydensified HA filler (Esthélis® Basic); b non-cohesive HA filler (Restylane®). Large amorphous material and electron-rich zones are shown at higher magnifications in insets. C = Collagen; F = fibroblasts; G = HA gels. Scale bars = 1.5 μm.
in all analysed skin sections suggests absence of immune response with regards to the exogenous implants. Furthermore, no granuloma formation has been observed in any of the tested conditions, confirming the safety of these dermal fillers. These results are well aligned with clinical studies confirming the safety of the tested HA fillers, demonstrated by absence or mild transient adverse reactions [3, 13–16, 19].

Alcian blue and colloidal iron stainings were used to visualize exogenous HA fillers (fig. 1). Both stainings showed similar results. The difference in the intensity of the stainings should reflect higher local concentration of HA in the non-cohesive filler (Restylane®) as compared to the cohesive polydensified (Esthélis® Basic) and cohesive monodensified (Juvederm® Ultra) fillers. A decrease in the intensity of the staining from 8 to 114 days observed in all tested fillers suggests a decrease in the amount of HA, and thus its progressive degradation. HA fillers are initially distributed within the skin at the moment of injection. In our study all fillers showed a tendency to agglomerate, probably due to mechanical forces caused by mobility of the skin tissue over time. Nevertheless, we did not observe a major redistribution within the dermis after 114 days.

Observations of tested fillers with respect to the extent of their distribution are consistent with van Gieson elastin staining results. The cohesive polydensified filler Esthélis® Basic is distributed homogeneously within the dermis, suggesting most natural bio-integration (fig. 2b, f). It spreads throughout the dermis by filling even the smallest spaces between collagen bundles. This observation is coherent with the fact that the gel consists of higher- and lower-density zones, allowing it to optimally integrate within the dermis. The cohesive monodensified filler Juvederm® Ultra forms small aggregates and is thus distributed less homogeneously among collagen bundles and elastin fibres after 8 and 114 days (fig. 1c, g; fig. 2c, g). However, it does not displace them as the non-cohesive filler Restylane® (fig. 2d, h). The latter forms larger aggregates that shift collagen and elastin fibres resulting in the least homogeneous HA distribution after 8 and 114 days. Despite these differences the contact between dermal fillers and collagen/elastin fibres does not induce any morphological alterations of the latter in any of the studied conditions, nor does it impact their functionality to absorb the staining dyes.

Remarkably, the pattern of differences observed in the bio-integration of the tested fillers in the reticular dermis was constant between 8 and 114 days. Presence of HA fillers in the injected area of the dermis after 114 days demonstrates that resorption of all HA fillers is a slow and progressive process.

Flynn et al. [17] conducted a similar study and analysed histological sections of various HA formulations immediately after injection. The biopsies were taken 3 min after injection on the same day, and 14 days after injection to focus evaluation on the early reaction. In this respect, the two studies are complementary and, interestingly, report very similar results, both in terms of how the fillers are distributed within the skin, and in terms of their tolerability.

Quan et al. [20] showed that a non-cohesive (biphasic) HA filler injected into the dermis of individuals over 70 years old stimulates procollagen I synthesis by mechanical forces that stimulate fibroblasts by stretching dermal structures. We did not observe an increase in procollagen I in any of the gels in our study, presumably because we did not inject the filler into a photo-exposed and aged skin (>70 years), since the mean age of our volunteers was 52 years.

Observation of skin samples treated with Esthélis® Basic and Restylane® using TEM confirmed that HA injection did not alter the structure of fibroblasts, collagen and elastin fibrils, as well as extracellular matrix components (fig. 3), corroborating our histological results. Morphological conservation of fibroblasts is particularly important, as they are responsible for most of the collagen and elastin synthesis, as well as renewal of extracellular components [21]. Abundance of cytosolic vesicles and rough endoplasmic reticulum demonstrated conservation of cellular functionality and continuous protein synthesis. A surprising feature of TEM micrographs was the presence of electron-dense dots and filaments in areas infiltrated by HA fillers. Since HA cannot be visualized by conventional staining for TEM, we hypothesized that these electron-dense structures correspond to the proteoglycan network of the extracellular matrix that is infiltrated by HA gels.

**Conclusion**

Considering the high demand for soft tissue augmentation procedures and a vast range of HA fillers that exist on the market, safety remains a paramount requirement for all products. Our results after 8 and 114 days showed that regardless of manufacturing technologies all tested dermal fillers preserve skin morphology, do not induce immune response, and therefore have excellent tolerability. Absence of adverse reactions in this study, supported by the published literature, further confirms the safety of the tested HA dermal fillers.
Different cross-linking technologies result in HA dermal fillers with specific characteristic visco-elastic properties which, in consequence, behave differently when injected into human skin. Histological results demonstrated that soft tissue integration is dependent on the nature of the dermal filler. The cohesive polydensified filler Esthélis® Basic showed the most homogeneous distribution when injected intradermally. Its optimal spreading within the reticular dermis is achieved by filling even the smallest spaces between collagen bundles and elastin fibrils, while preserving the structural integrity of the latter. Therefore, appropriate fillers for desired aesthetic applications can be selected based on their characteristic bio-integration related to their specific physical properties.

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