Elucidations on the Performance and Reversibility of Treatment with Hyaluronic Acid Based Dermal Fillers: In vivo and in vitro Approaches

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Purpose: The aim of this study was to investigate the performance and the reversibility of different classes of Hyaluronic Acid (HA) dermal fillers. We analysed 4 HA based fillers, belonging to 3 different chemical classes of products, commonly used in the field of wrinkles correction: linear HA 8 mg/mL (Viscoderm 0.8), thermally stabilized hybrid complexes of high and low molecular weight HA molecules at a concentration of 32 mg/mL and 45 mg/mL respectively (Profhilo and Profhilo Structura) and cross-linked HA 25 mg/mL (Aliaxin GP).

Methods: The products were tested by a well-established animal model. The generated implants were analyzed through High-Frequency Ultrasound technology. Then, reversibility of the treatment was evaluated by enzymatic degradation kinetics studies, characterised by a combined approach of Carbazole assay and HP-SEC/TDA method.

Results: Implants generated by linear HA 8 mg/mL remained detectable by ultrasound acquisition for 4 weeks, whereas those generated by injection of HA hybrid complex 32 mg/mL were detectable for 10 weeks. HA hybrid complex 45 mg/mL and cross-linked HA 25 mg/mL were detectable for 29 and at least 33 weeks, respectively. Enzymatic degradation kinetics studies demonstrated that the HA content in HA hybrid complex 45 mg/mL was almost completely depolymerized and homogeneous after 3 h of treatment. For cross-linked HA 25 mg/mL, 24 h of incubation are needed to obtain the same degree of depolymerization.

Conclusion: The study confirmed the ability of the experimental model to predict the behaviour of HA based dermal fillers in vivo and showed the innovative aspects of HA hybrid complex 45 mg/mL, that combines the high-safety profile, in terms of reversibility of the treatment, of the linear HA-based products with the durability of a high degree cross-linked gels, paving the way to the chance to be used for a wide range of applications in the field of aesthetic medicine.

Keywords: hyaluronic acid, dermal filler, crosslinking, NAHYCO, high-frequency ultrasound, anti-aging, hyaluronidase, reversibility

Introduction

The clinical practice in aesthetic and regenerative medicine has widely recognized the value, in terms of effectiveness and safety, of Hyaluronic Acid (HA) based fillers. Devices containing HA are commonly used in dermal rejuvenation field to treat wrinkle correction or to volumize specific face areas, in addition, interesting biomedical applications are consolidating (intraarticular disorders, urinary tract dysfunctions, bacteriostatic activity etc). The form of the HA molecule predominantly used in the majority of common dermal fillers is that of a biocompatible and biodegradable glycosaminoglycan (GAG). It consists of repeats of disaccharide units of glucuronic acid and N-acetylglucosamine, commonly used with a molecular weight (MW) range between 200 to 2000 kDa.

Often HA is also employed as a cross-linked polymer obtained by the chemical linking of an HA polymer chain to each other. This chemical reaction is promoted by adding external chemical agents (such as 1,4-butanediol diglycidyl...
ether (BDDE)) and permits to significantly modify and modulate the physical properties of the final products, making them longer-lasting and less likely to be degraded, delaying the need of a subsequent injection.

Another interesting patented technology, called NAHYCO Hybrid Technology, launched by IBSA in 2015, offers a new physico-chemical scenario to HA based fillers, promoting interactions between long and short HA monomers, without changing the disaccharide unit structure and without introducing other chemical compounds. The final formulation obtained is a hybrid cooperative complex that offers new features in terms of stability and longevity of the final products, enhancing the safety at the same time.

Therefore, the modulation of the number of the disaccharide units of HA monomers (ie MW), the grade of cross-linking between HA molecules and their molar concentration and mixing (hybrid complexes), offer to the HA based fillers variability in terms of physico-chemical characteristics that consequently influences the rheology of the final product (elastic modulus G’, viscous modulus G”, complex modulus G*cohesivity, Tan δ etc.).

Consequently, there is a marked versatility in the use of the product in the biomedical field. Suffice is to say that minimal variations in the above listed parameters allow to tailor the specific medical indication of HA fillers.

To date, the research in this area continues to offer clarifications on all those biophysical and biological mechanisms that chorally influence the performance and safety of such devices. Given the increasing clinical interest, nevertheless, further investigations are needed to lead the design of new formulas with increasing modular performances in terms of use and durability.

Biophysical characterization, in vitro studies and clinical observations offered precious information about filler behaviour in human dermis, but new robust data, net of possible speculation, are needed to allow increasing precise customisation of fillers formulation.

Actually, the use of analytical methods that allow greater overview represents an added value in this type of study, allowing the researchers to consider in the final result of a specific treatment also the contribution provided by the physiological pleiotropic response of the body, as well as the chemical-physical properties of the product.

In this regard, our research team developed a valid non-invasive, sensitive and reproducible in vivo analytical tool based on the High-Frequency Ultrasound (HF-US) imaging technology. This method is able to preliminary screen new HA based formulations, specifically evaluating their residence time after subcutaneous injection in mice.

HF-US could represent a fast and innovative technology to obtain high-resolution images of the skin, of the underlying tissues, and, eventually, of the subcutaneously injected compounds in an inexpensive and non-invasive manner.

Using this innovative methodology, in the present study we investigated the performance, in terms of longevity after subcutaneous injection, of a library of 4 HA fillers, belonging to different classes of compounds: linear compounds, hybrid cooperative complexes based compounds (NAHYCO) and cross-linked compounds.

Even if the injection of HA based dermal fillers is an increasingly popular aesthetic practice, potential complications due to inappropriate or unpredictable clinical administrations could happen (eg unaesthetic overcorrections, vascular occlusions etc.).

For this reason, the need of reversible filler injections is particularly coveted.

Clinical practice suggests the use of hyaluronidase to manage these types of complications linked to dermal fillers administrations. Indeed, the timely infiltration of hyaluronidase leads to the degradation of HA fillers, rescuing from more severe vascular complications.

Some chemical-physical parameters of HA based fillers as HA concentration, degree of cross-linking, cohesive properties etc. affect the efficacy of the hyaluronidase activity and, consequently, the reversibility of HA based filler.

Considering what said, a careful analysis to assess in vivo performance of the tested compounds, complemented by the in vitro analysis of their reversibility, provides useful insights on the impact of chemical and physical features on the global performance and safety of HA based fillers and, consequently, paved the way to the design of novel highly performing, tailored and biocompatible HA based fillers.
Materials and Methods

Hyaluronic Acid Products

All the formulations tested in this study, Viscoderm 0.8 (linear HA with a molecular weight of about $1 \times 10^6$ Da; concentration of HA: 0.8% w/v, 8 mg/mL), Profhilo (hybrid cooperative complex-based compound with low (L-HA) and high (H-HA) molecular weight HA in a ratio 1 to 1; concentration of HA: 3.2% w/v, 32 mg/mL, molecular weight of H-HA 1.4 – $2.1 \times 10^6$ Da, molecular weight of L-HA 65–110 KDa), Profhilo Structura (a hybrid cooperative complex-based compound containing low (L-HA) and high (H-HA) molecular weight HA in a ratio 1 to 1; concentration of HA: 4.5% w/v, 45 mg/mL) and Aliaxin GP (cross-linked HA with molecular weight from 1000 kDa to 2000 kDa, concentration of HA: 2.5% w/v, 25 mg/mL) were from IBSA Farmaceutici Italia (Lodi – IT). Each product analysed will be identified in accordance with an internal nomenclature as reported in Table 1.

Animals

The animal protocol (no 4547) was approved by the Italian “Ministero della Salute”; all the experimental procedures were performed according to Italian guidelines and regulations. CD1 mice (female, 8–10 weeks, not pregnant) were purchased from Charles River Laboratories. Prior to the experiments, animals were housed for 7 days for acclimatization to animal room conditions.

Experimental Groups

A total of 25 mice were used, allocated in five experimental groups with five animals in each, as described in Table 1. Saline solution was used as negative control.

Administration Procedures and Image Acquisition Through HF-US Imaging Technology

Administration of hyaluronic acid compounds and image acquisition were performed as previously described. Briefly, all mice were shaved in the dorsal region and disinfected. A subcutaneous injection of 200 µL of each compound was given paraspically along the dorsum of each mouse on each side of the vertebral column, taking care to ensure equal injection distance and consistent injection shape. All procedures described were performed under general anaesthesia and sterile conditions. The volume occupied by the hyaluronic acid compounds under the mouse skin was measured through the HF-US imaging technology with the VisualSonics Vevo™ 2100 In Vivo High-Resolution Micro-Imaging System (VisualSonics Inc., Toronto, ON, Canada) equipped with a three-dimensional (3D) motor on a rail system. This equipment allowed to acquire a series of consecutive two-dimensional (2D) images of the subcutaneous implants. Successively, drawing the ROIs (Regions Of Interest) on the sequence of 2D images collected during the scan enabled the Vevo 2100 3D image software to generate 3D volumetric sonograms and to calculate the total volume of the implant.

In vitro Enzymatic Degradation

In vitro enzymatic degradation studies were conducted on HA hybrid complex 45 mg/mL and cross-linked HA 25 mg/mL.

Table 1 Experimental Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline Solution</td>
</tr>
<tr>
<td>2</td>
<td>Linear HA 8 mg/mL (Viscoderm 0.8)</td>
</tr>
<tr>
<td>3</td>
<td>HA hybrid complex 32 mg/mL (Profhilo)</td>
</tr>
<tr>
<td>4</td>
<td>HA hybrid complex 45 mg/mL (Profhilo Structura)</td>
</tr>
<tr>
<td>5</td>
<td>Cross-linked HA 25 mg/mL (Aliaxin GP)</td>
</tr>
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</table>
The soluble fractions of both products were determined and characterised.

HA content analysis and the molecular weight evaluation (number-average molar mass Mn, weight-average molar mass Mw, Mw/Mn) were performed respectively by carbazole assay and by the Size Exclusion Chromatography–Triple Detector Array (SEC-TDA) equipment.

To evaluate enzymatic degradation, 1g of each product was diluted with 20.0 mL of a final solution of hyaluronidase (8U/mL in PBS) from bovine testes (type I-S, lyophilized powder, Sigma-Aldrich) at 37°C under stirring. At different incubation times (0.5h, 1h, 3h, 24h, 48h) the samples were diluted with PBS, filtered using a 0.2 µm RC filter and heated to 100°C for 5 min to inactivate the enzyme. The obtained sample stock solutions were then diluted in order to quantify the HA content by the carbazole assay and to determine the molecular weight by SEC-TDA.

The degradation was monitored by following the trend of the HA soluble fraction amount and by evaluating its average molecular weight during enzymatic degradation.

HA Content Determination (Carbazole Assay)
The carbazole assay was performed as described in the European Pharmacopeia (EP) monograph of “Sodium Hyaluronate”.

The concentration of the soluble HA in the products was calculated as follows.

\[
\text{soluble HA concentration (mg/g)} = \frac{C_{\text{st}} \cdot \text{Abs}_{\text{st}} \cdot K \cdot w \cdot 401.3}{194.1}
\]

where: \(C_{\text{st}}\) = concentration of the standard solution (mg/mL), \(\text{Abs}_{\text{st}}\) = absorbance of the standard solution, \(\text{Abs}_{\text{sa}}\) = absorbance of the sample solution, \(K\) = dilution factor (mL), \(w\) = sample weight (g), 194.1 = molecular weight of glucuronic acid (g/mol), 401.3 = molecular weight of the disaccharide fragment (g/mol).

The soluble fraction % of HA in the product was determined by the following formula:

\[
\text{HA soluble fraction (％)} = \frac{[\text{soluble HA in the products}]}{[\text{labeled HA in the products}]}
\]

HA Average Molecular Weight and Molecular Weight Distribution by HP SEC/TDA Method
Data were recorded with Omnisec Chrome software (version V11.31 Malvern Panalytical, UK) with a refractive index (RI) increment (\(\delta n/\delta c\)) of 0.155.\(^{26}\)

The chromotographic technique employed allows quantification of HA content and an estimate of its average molecular weight in each sample analyzed. In detail, RI, low angle light scattering (LALS), right angle light scattering (RALS) and pressure difference (DP) spectra were elaborated to obtain recovery (%), Mn, Mw and polydispersity index (PI) of HA. The larger the PI, the broader the molecular weight distribution of a polymer. A monodisperse polymer where all the chain lengths are equal has PI = Mw/Mn = 1.

The instrument consisted of liquid chromatography system and triple detector (Omnisec, Malvern Panalytical Ltd., Malvern, UK) equipped with pump and thermostatic oven for chromatographic column. The set of two A6000M columns, 1.3 µm, 300 × 8.0 mm, (Viscotek, Malvern, UK) were used with a flow rate of 0.6 mL/min, runtime analysis of 60min and elution temperature of 40°C. The mobile phase was composed by 0.1 M sodium nitrate and 0.05% sodium azide and the injection volume was 100 µL. Detector parameters (RI), Viscometer and Light scattering were measured at 90°C and 8°C.

Results
Performance in vivo
The aim of this work was to characterize the performance, in terms of longevity and permanence, of different classes of dermal filler made with hyaluronic acid after subcutaneous injection. For this purpose, we used a well-established animal model and an innovative and not invasive method for the acquisition of experimental data, previously published by our
Briefly, before the administration procedure, the dorsal region of the mice was shaved and disinfected. Then, the formulations of interest were injected subcutis on each side of the vertebral column of each mouse, in order to obtain two subcutaneous implants (one implant per flank) in the caudal region of the dorsum. Immediately after compounds injection, the volume of the generated implants was measured by high frequency ultrasound (HF-US), through the VisualSonics Vevo 2100 Imaging Station system as described in Methods. Compounds injected subcutis appeared as ovoid ipoechogenic (black) mounds just below the line (white/grey) of the skin. After the first image acquisition, the residence of the subcutaneous implants was monitored, through the HF-US system, for all the experimental groups until the disappearance of the signal for a maximum of 33 weeks. Then, considering the slow kinetics of degradation of the implants still detectable, image acquisitions were performed less frequently.

As reported in Table 2 and shown in Figure 1, the formulation containing linear hyaluronic acid molecules, linear HA 8 mg/mL, had a detectable ultrasound signal only until the 4th week, showing the fastest kinetics of degradation. The volume of the implant generated by the injection of HA hybrid complex 32 mg/mL, containing hybrid cooperative complexes with low and high molecular weight HA, instead, was subjected to a little decrease in the first 4 weeks (only about 20% respect to day 0) and remained detectable by ultrasound until the 9th week. Unexpectedly, HA hybrid complex 45 mg/mL, similar to HA hybrid complex 32 mg/mL in terms of composition but with a higher concentration of hyaluronic acid, showed a very slow volumetric degradation. In fact, in the first 3 weeks of monitoring we did not observe a decrease in the volume of the subcutaneous implants generated by the injection of HA hybrid complex 45 mg/mL. In addition, until the 8th week the volume of the implants decreased of only about 20% respect to the day of injection and the permanence of the compound was detectable until the 27th week. Finally, the implants generated by the injection of cross-linked HA 25 mg/mL showed the slowest volumetric degradation, remaining detectable by ultrasound imaging until the 33rd week. These data are consistent with existing literature data on chemically modified dermal fillers, that are known to be more resistant to enzymatic degradation by hyaluronidases and able to assure a high durability of the aesthetic effect.

It is noteworthy the transient increase of the volume of the implants registered in the first week post injection. This effect could be due both to a strong hydration of the exogenous HA molecules that, absorbing water from the surrounding tissues, increased their volume and to an inflammatory response. In the gross observation of the treatment sites, they did not show other typical clinical signs of cutaneous inflammatory reactivity; nevertheless, being cross-linked HA 25 mg/mL a formulation containing chemically modified molecule, it is not possible to completely exclude the possibility that the treatment induced an inflammatory response and edema. However, this temporary phenomenon was considered not biologically relevant because the safety of the present product was internally investigated by the standard tests for biocompatibility evaluation of medical devices.

Finally, it is important to highlight the macroscopic differences noted about the visual aspect of the implants generated by the injection of HA hybrid complex products and cross-linked HA 25 mg/mL, though attention was taken to assure the same shape of the implants during the administration procedure. In fact, immediately after injection,

**Table 2 Volumetric Degradation of the Subcutaneous Implants, Expressed as Percentage Respect to the Day of Injection**

<table>
<thead>
<tr>
<th></th>
<th>Weeks Post Injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Linear HA 8 mg/mL</td>
<td>100</td>
</tr>
<tr>
<td>(Viscoderm 0.8)</td>
<td></td>
</tr>
<tr>
<td>HA hybrid complex 32 mg/mL (Profhilo)</td>
<td>100</td>
</tr>
<tr>
<td>HA hybrid complex 45 mg/mL (Profhilo Strucutra)</td>
<td>100</td>
</tr>
<tr>
<td>Cross-linked HA 25 mg/mL (Aliaxin GP)</td>
<td>100</td>
</tr>
</tbody>
</table>
both HA hybrid complex products and cross-linked HA 25 mg/mL led to the appearance of subcutaneous ovoid bulges, like real *mounds*. Nevertheless, in the following weeks, bulges produced by HA hybrid complex products disappeared, leaving a mild swelling and turgor in the treatment site, despite the permanence of a well-defined ultrasound signal. On the other hand, mounds produced by the injection of cross-linked HA 25 mg/mL remained visible at the naked eye until the last week of the study.

**In vitro Degradation results**

HA hybrid complex 45 mg/mL and cross-linked HA 25 mg/mL were further analyzed by in vitro degradation approach, in order to discriminate the potential reversibility of the treatment with these products.

To detect and significantly discriminate the degradation kinetics of HA hybrid complex 45 mg/mL and cross-linked HA 25 mg/mL after treatment with hyaluronidase, the presented in vitro enzymatic procedure employed a hyaluronidase concentration of 8U/mL.

As showed by results summarized in Table 3, HA hybrid complex 45 mg/mL contained only free soluble HA and its average molecular weight decreased regularly during in vitro degradation study. On the other hand, cross-linked HA 25 mg/mL, according to its cross-linked nature, contained around 13% of soluble HA fraction. During enzymatic degradation, its soluble fraction increased up to 70–74% reaching a plateau after 1 hour. Only once the soluble fraction plateau was reached, the average molecular weight began to decrease. Figure 2 depicts a graphical representation of the degradation kinetics profile of the two products.

After 24h the average molecular weights of the two products became comparable. The delay of the molecular weight degradation of the cross-linked gel if compared to the free HA gel can be explained by considering the Mark-Houwink plot for each time point during the incubation.30,31

Figure 3A depicts the Mark Houwink plot of cross-linked HA 25 mg/mL over the incubation time; it is almost evident how the trend became linear as the degradation continued.

During the first hour of incubation the hyaluronidase activity tended to degrade both the existent HA soluble fraction and the cross-linked HA, thus increasing the soluble amount of HA with an irregular trend of its average molecular weight. Once the soluble fraction reached a plateau, the enzyme activity focused only on its degradation and the molecular weight decreased rapidly.
In the case of HA hybrid complex 45 mg/mL, the whole amount of HA was free, soluble and already available for the enzymatic activity at the beginning of the degradation. The Mark-Houwink plot for HA hybrid complex 45 mg/mL over the incubation time is represented in Figure 3B.

Focusing the attention on the average molecular weight distribution, in Figures 4A and B the RI graphs of the products soluble fractions over the incubation time are reported.

### Table 3 Summary of the Main Degradation Data of HA Hybrid Complex 45 mg/mL and Cross-Linked HA 25 mg/mL

<table>
<thead>
<tr>
<th>Product</th>
<th>Degradation Time (h)</th>
<th>Carbazole Assay</th>
<th>SEC/TDA Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soluble HA (%)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Cross-linked HA 25 mg/mL (Aliaxin GP)</td>
<td>0</td>
<td>11.5</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>45.7</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>70.0</td>
<td>72.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>72.8</td>
<td>74.4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>73.2</td>
<td>73.9</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>71.3</td>
<td>72.8</td>
</tr>
<tr>
<td>HA hybrid complex 45 mg/mL (Profhilo Structura)</td>
<td>0</td>
<td>97.6*</td>
<td>101.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>-</td>
<td>101.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>100.8</td>
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<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>99.4</td>
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<tr>
<td></td>
<td>24</td>
<td>-</td>
<td>100.2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>100.4</td>
</tr>
</tbody>
</table>

**Notes:** *Since the soluble fraction of HA hybrid complex 45 mg/mL was around 100% before the degradation, during the enzymatic reaction the HA content analysis was performed only on the cross-linked gel samples.*

In the case of HA hybrid complex 45 mg/mL, the whole amount of HA was free, soluble and already available for the enzymatic activity at the beginning of the degradation. The Mark-Houwink plot for HA hybrid complex 45 mg/mL over the incubation time is represented in Figure 3B.

Focusing the attention on the average molecular weight distribution, in Figures 4A and B the RI graphs of the products soluble fractions over the incubation time are reported.

**Figure 2** Graphical representation of the degradation kinetics profile of HA hybrid complex 45 mg/mL and cross-linked HA 25 mg/mL. At the top-right, an enlargement of the first 15h of incubation is reported.
As illustrated in Figure 4A, at $t_0$ HA hybrid complex 45 mg/mL shows two different peaks, the first related to the HA with high molecular weight (HMW-HA), and the second one related to the HA with low molecular weight (LMW-HA). The whole soluble fraction is represented by the area under the curve. According to the Recovery % and the carbazole assay results (Table 3) it is evident how all the HA in the product was free and soluble. As the degradation time passed, the peak related to the HA with high molecular weight (HMW-HA) disappeared, generating a single peak that shifted to higher retention volumes (lower average molecular weights) and became sharper, thus demonstrating how, during degradation, the decrease in Mw was closely related to a reduction of the PI.

As depicted in Figure 4B, the soluble fraction of cross-linked HA 25 mg/mL at $t_0$ shows a single, small and broad peak. According to the Recovery % and the carbazole assay results (Table 3), only a 13% of the labeled amount of HA was comprised in the soluble fraction of the product.

The area under the curve increased during incubation. After 3h, the peak became sharper and shifts to higher retention volumes, as observed for HA hybrid complex 45 mg/mL.
Discussion

As widely discussed in the introduction, HA based dermal fillers are among the most used remedies for wrinkle correction and soft tissue rejuvenation, due to their non-invasiveness and high-safety profile. In addition, in the recent years, companies were encouraged to develop lines of products able to respond to all the different requirements and guarantee a complete restoration treatment in place of a single product for a specific need.

In this work, we tested 4 HA products, belonging to three different classes of compound, in order to investigate their behavior after subcutaneous injection, mainly in terms of durability.

Linear HA 8 mg/mL is a linear hyaluronic acid-based product and it is part of a wider line of products specifically indicated for the correction of the facial defects. Being the concentration of the hyaluronic acid quite low, Linear HA 8 mg/mL is indicated in the maintenance session of the treatment. As known, linear hyaluronic acid can be easily subjected to the action of the hyaluronidases and rapidly undergoes enzymatic degradation. Indeed, in our investigation, results obtained with Linear HA 8 mg/mL are consistent with literature data and clinical application, showing the fastest degradation kinetics. The clinical effectiveness of HA hybrid complex 32 mg/mL and cross-linked HA 25 mg/mL is well-documented. The stabilization of the mixture of the hybrid complexes through the NAHYCO technology led to the generation of a unique product that has low viscosity and high manageability despite the high concentration of hyaluronic acid and a durability after injection similar to that of weakly cross-linked products without the inflammatory
responses often associated with the latter. In addition, HA hybrid complex 32 mg/mL was proven to homogeneously expand adipose compartments and integrate into tissues, favoring the remodeling of the extracellular matrix in terms of elasticity and support. The same technology allowed to develop HA hybrid complex 45 mg/mL, a hybrid complexes based product with a higher concentration of hyaluronic acid. Surprisingly, in our experimental animal model, HA hybrid complex 45 mg/mL showed a durability after subcutaneous injection similar to that of cross-linked HA 25 mg/mL, a product with a high degree of cross-linking. These results confirm the innovative aspect of the NAHYCO technology, that with a low increase in the concentration of hyaluronic acid led to a huge increase in the durability of the product. Moreover, the macroscopic observation of the treatment site allowed us to confirm the high plasticity and adaptability to surrounding tissues of HA hybrid complex products and, specifically, of HA hybrid complex 45 mg/mL. In fact, the bulges generated immediately after the injection disappeared yet in the second week after the administration, however increasing the turgor of the treated tissues and achieving the desired “filling effect”.

The management of dermal filler complications, caused by inappropriate injections, by the use of hyaluronidase, is consolidated in the clinical practice. The amount of hyaluronidase commonly injected could vary between 15 to 200 U/mL, according to the chemical-physical properties, such as the concentration of HA in the filler, the degree of cross-linking or cohesive properties of the filler to degrade and the complexity of the side effects. This means that, in most cases, the quality of the performance of HA based dermal fillers is negatively related to the reversibility of the treatment.

For this reason, as secondary end point of the present work, we investigated the reversibility, evaluated in terms of resistance to the degradation action of hyaluronidase, of more durable fillers identified in this study, HA hybrid complex 45 mg/mL and cross-linked HA 25 mg/mL.

The data obtained through an in vitro enzymatic degradation assay suggest that HA hybrid complex 45 mg/mL is more sensitive to the enzymatic activity of hyaluronidase respect to cross-linked HA 25 mg/mL. This characteristic is particularly important, in terms of safety, when a rapid action of hyaluronidase treatment, after an inappropriate injection, is required. In detail, HA hybrid complex 45 mg/mL was almost completely depolymerized and homogeneous after 3 h of treatment while for cross-linked HA 25 mg/mL, 24 h of incubation are needed to obtain the same degree of depolymerization (Figure 2).

The correlation between in vivo permanence data with in vitro enzymatic degradation results shows that HA hybrid complex 45 mg/mL evidences a comparable duration in vivo respect to the cross-linked HA 25 mg/mL filler (29 weeks vs 33 weeks, respectively) but, at the same time, an interesting more degradability by hyaluronidase, mainly at the early stage of the in vitro kinetics study (Figure 2).

Our analyses demonstrate that NAHYCO technology is peculiar to guarantee, simultaneously, comparable performance and a higher degree of safety, expressed as reversibility rate, than cross-linking technology.

This means that HA hybrid complex 45 mg/mL, thanks to its high-safety profile, durability and overall versatility paves the way to a new manner of treatment of skin defects, being able to provide a solution that answer to the requirements of a complete restoration treatment in place of the system of “a single product for a single wrinkle”.

**Conclusion**

In conclusion, this study not only confirmed the ability of our experimental model to predict the behavior, in terms of appearance and residence time, of hyaluronic acid based dermal filler after injection but also showed the innovative and advantageous aspects of the NAHYCO technology. Specifically, it demonstrated how, as a result of a small increase in hyaluronic acid concentration, this technology obtained a new and very promising product, HA hybrid complex 45 mg/mL, that, though preserving the high safety profile and the natural aesthetic effect of a classic linear hyaluronic acid based dermal filler, has the durability similar to that of a high degree cross-linked gel.

**Disclosure**

Mario Scrima, Filomena Merola, Nicoletta Vito, Daniele Pacchioni, Gabriele Vecchi, Carmela Melito, Antonio Iorio, Andrea Maria Giori and Angela Ferravante are employees of IBSA Farmaceutici Italia, a pharmaceutical company based in Lodi, Italy. Andrea Maria Giori reports personal fees from IBSA Farmaceutici Italia, during the conduct of the study. The authors report no other conflicts of interest in this work.
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