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MORPHOLOGICAL EVIDENCES FOLLOWING PEGYLATED FILLER TREATMENT IN HUMAN SKIN

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In this study, microscopic, histochemical and ultrastructural observations in human skin are presented, 8 months after an injection of a novel PEGylated filler. Morphological features demonstrated an excellent integration of the filler with the connective tissue components and an effective interpenetration with the ground substance. The filler appears uniformly distributed inside the hypodermis. No segregation or encapsulation of cells and other structures was observed nor evidence of immunological adverse reaction. Furthermore, observed ultrastructural modifications of fibroblasts supports a stimulatory effect of molecular components production of the extracellular matrix, contributing to the cutaneous connective tissue renewal.

Hyaluronic Acid (HA) based fillers, due to highly appreciated structural, rheological and physiological properties are widely used in cosmetics, biomedicine and aesthetic medicine for the correction of ridges and for the restoration of skin volumes. HA is characterized by high moisturising retention ability and viscoelasticity, water balance, flow resistance, capability as a lubricant and as a stabilizing agent for connective tissues. Furthermore, HA is lacking of toxicity and immunogenicity.

The first highly purified HA, extracted from avian umbilical cord and rooster comb, was obtained and patented in 1979 (1). In the following years, other HA based hydrogels of animal origin, suitable for supplementation into the connective tissue of the skin, were introduced and developed (2, 3, 4), and the extractions of HA of animal origin were then substituted by production of microbial fermentation by *Streptococci* (5). However, due to pathogenic source (bacterial DNA residuals and/or some peptide remnants from industrial production), HA produced in this way would be immunogenic and its use would be risky.

In the following years, a more advanced technique permitted the production of non-immunogenic HA with recombinant *Bacillus subtilis* on an industrial scale (6). Modifications of HA concentration and degrees of cross-linkage with biphasic and monophasic gels were experimented (7). Crosslinking agents were also introduced and changed in order to decrease the toxicity and to increase both biocompatibility and long lasting effectiveness. In this way, after divinyl sulfone and 1.4-butanediol diglycidyl ether, polyethylene glycol (PEG) polymer was introduced. PEG polymer, used as a crosslinking agent creating the so-called

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PEGylation (8, 9, 10), seemed to offer considerable advantages in terms of safety and performance of the gel.

Both PEG and HA are polymers and their cross-linkage allows to create matrices with scaffold structure (11, 12, 13), as a 3D web constituted by interpenetrated knots and links, thus offering a better integration of the filler into the connective tissue. Furthermore, such a structure would permit the retaining and gradual releasing of molecules suitable for skin rejuvenation.

On this basis, we have chosen for this study a new HA-based filler, in which HA was obtained by fermentation from a bacterial strain of *Bacillus subtilis* (non pathogenic, belonging to the class of probiotics). It was complexed with PEG through a process of cross-linkage of the two polymers (PEGylation), forming a 3D molecular scaffold for a better integration with the connective tissue components, a long lasting filling effect and a better resistance of the skin to thermal and mechanical stress.

MATERIALS AND METHODS

Five volunteers received a subdermal injection of a small volume (1 ml) of PEGylated hyaluronic acid based filler (Neauvia Stimulate®) into the hypodermis and 8 months later skin biopsies were carried out.

Light microscopy

Biopsy specimens were directly immersed in the fixing solution 4% paraformaldehyde/phosphate buffer for 24 h and then processed for light microscopy (dehydrated, embedded in paraffin, and sectioned). Seven µm-thick sections were stained with Hematoxylin and Eosin (H&E), other adjacent sections were stained 30 min with Alcian blue solution 1% in 3% acetic acid. Microscopic observations were made with Carl Zeiss Axioplan microscope equipped with a Nikon DS-Fi2 high definition 5-megapixel colour CCD camera head.

Electron microscopy

Fixation was performed by immersion of bioptic samples in a 2.5% glutaraldehyde - 2% paraformaldehyde in 0.1M sodium cacodylate buffer solution (pH 7.3) for 6 h at 4°C. Samples were post-fixed for 2 h in 1.33% osmium tetroxide in 0.1M s-collidine buffer and dehydrated in a graded series of ethanol. Finally, specimens were embedded in epoxy resin Epon 812 for sectioning. Ultra-thin (40-60 nm) sections were obtained at the ultramicrotome Reichert Ultracut S provided with a Diatome diamond knife. Semi-thin sections (0.2 µm) obtained for selection of interest areas were stained with Toluidine blue and observed with a Zeiss Axioplan microscope provided with specific filters for differential interference contrast.

Ultrathin sections, after the collection on 200 mesh grids, were counterstained with lead citrate and uranyl acetate. Observations and electron micrographs were made at a transmission electron microscope Zeiss EM 10 operating at 80 kV with an objective aperture of 30/60 µm. Images were recorded on Kodak 4489 Electron Image film and finally digitized with an Epson Perfection V750 Pro scanner at 1200 dpi.

RESULTS

In sections of the biopsy specimens observed by light microscope, the different layers of skin are well detected in the general view at low magnification: epidermis (EP), dermis (D) and hypodermis (HD) (Fig. 1).

In the section stained with H&E (Fig. 1, HE), the diffused eosinophily (reddish) in the dermis is due to the abundance of small/medium sized bundles of collagen fibres. In the hypodermis, bigger bundles of collagen fibres are intermingled with more clear areas differently shaped, often adjacent one another. These areas are showing a light-bluish appearance, due to a basophilic material mainly constituted by the filler. Some small and well marked rounded profiles (free or inside a white mass of adipose tissue), well visible in the center/middle and in the lower part of HE image, are representing sweat glands and excretory ducts.

The section represented in the Fig. 1Ab was stained with Alcian blue in order to stain acidic polysaccharides and glycosaminoglycans. In the dermis, the very faintly bluish appearance is due to the constitutive hyaluronic acid forming, together with proteoglycans, glycosaminoglycans

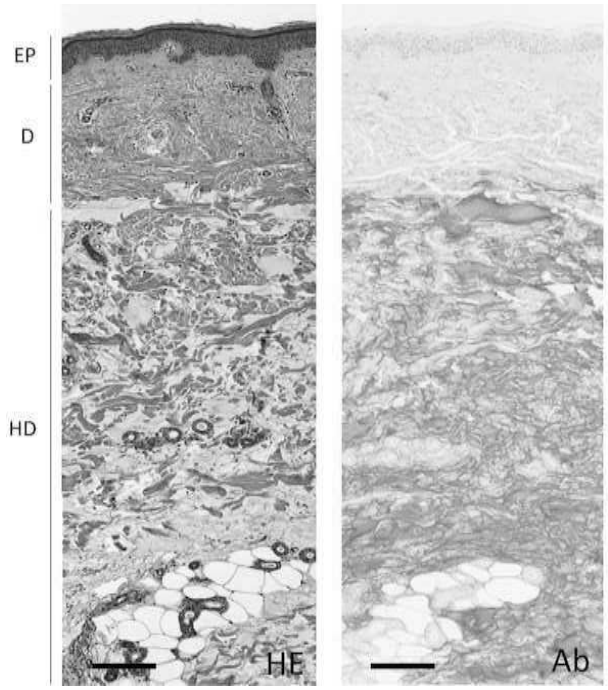

Fig. 1. *Skin sagittal section. Skin layers: epidermis (**EP**), dermis (**D**) and hypodermis (**HD**). **HE**: Hematoxylin and Eosin staining. The filler is shown as light blue areas between collagen fibres (red, in **D** and **HD**). Lower part of **HD**: clusters of adipocytes (**white**). **Ab**: Alcian blue staining. The filler (**blue**) among collagen fibres (not stained). Scale bars: 100 µm.*

and glycoproteins, the most part of the ground substance. In the hypodermis, Alcian blue intensely-stained material is particularly evident (Fig. 1Ab), realistically due to the high content of PEGylayed hyaluronic acid which was injected as filler in this part of the skin. Alcian blue, indeed, is specific for acidic polysaccharides and glycosaminoglycans, and therefore numerous areas appear specifically stained. These areas correspond to the ground substance and to the filler integrated with it; they are distributed between the collagen fibres, which are Alcian blue-negative. In the dermis, the very faintly cyan/bluish appearance is due to the constitutive hyaluronic acid, forming the most part of the ground substance, which was in part solubilized during the histologic preparative procedures. In Fig. 1Ab, particularly remarkable is the Alcian blue-positive intensely stained material in the hypodermis, due to the high content of PEGylated hyaluronic acid which was injected in this part of the skin.

In higher magnifications of different areas of hypodermis (Fig. 2), the filler stained by the Alcian blue appears blue and well recognizable, compacted or with elongated shape (Fig. 2a, b). These areas are constituted by a highly alcianophilic

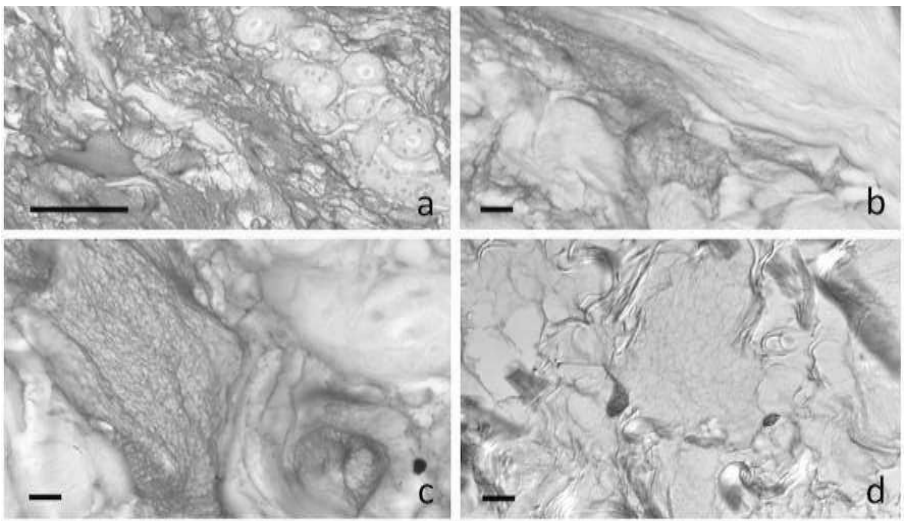

Fig. 2. ***a, b, c, d)*** *Alcian blue stained hypodermis sections. **a)** Components of a sweat gland (faintly stained round structures, on the right) surrounded by the filler (blue). The filler appears well integrated with hyaluronic acid of the ground substance. Scale bar: 100 µm. **b)** Collagen fibres (oblique structures not or faintly stained, on the top-right) in part covered by – and integrated with – the filler (blue). Scale bar. 10µm. **c)** Reticular appearance of Alcian blue stained material. Scale bar. 10 µm. **d)** Interference contrast microscopy imaging. Very fine texture of PEGylated HA as a reticular material, and a fibroblast with elongated cytoplasm are shown. Scale bar: 5 µm.*

material representing PEGylated hyaluronic masses distributed between bright collagen fibres, which appeared clear because not stained by Alcian blue. Inside these masses, well integrated within the subdermal connective tissue, it is possible to identify a fine texture constituted by highly stained filaments, arranged in a 3D network-like structure (Fig 2c, d). Between these areas, sweat glands and ducts transversaly sectioned were observable as very faintly stained circular structures (Fig. 2a).

Observing highly magnified alcianophylic structures by interference microscope, the network-like material shows the finely textured organization of the cross-linkage, where the hyaluronic acid is complexed with a PEG polymer forming a 3D network of variable mesh size (Fig. 2d, 3) with stabilization links between the two polymers showed as knots. This complex 3D structure (Fig. 3) related to the molecular architecture of interpenetrating polymer network, due to the very high extension of the molecular surface extremely rich of free polar groups, is well supporting the extraordinary capacity of binding huge amounts of water molecules contributing, in addition to the filler in itself, to increase the volume of the tissue.

The fine relationships between the different components of hypodermis and the material constitutive of the injected filler are also appreciable in sections stained with H&E (Fig. 4). In these sections, the injected material is showing a very light blue colour (Fig. 4a, b), and appears surrounding adipose tissue and intermingled with collagen fibres (Fig. 4a). Sweat glands and related ducts, due to the rounded profiles intensely stained, are clearly distinguishable (Fig. 4a). In Fig. 4b the filler material is directly and smoothly contacting collagen fibres without any incapsulation.

Fibroblasts have been also studied at the electron microscope in order to detect possible cytological modifications induced by the filler. Ultrastructural observations of a fibroblast are summarized as shown in Fig. 5. The nucleus appeared rich in dispersed chromatin (euchromatin, the active form of chromatin) and a big nucleolus (the site of synthesis of ribosomes, organelles for the synthesis of proteins) was detectable inside the nucleus. In the cytoplasm, a highly extended rough endoplasmic reticulum, with dilated cisternae containing a finely filamentous material, was evident. Another organelle, the Golgi apparatus, was highly represented in fibroblasts. It appears constituted by concave/convex stacks of membranous cisternae with associated vesicles.

In the living cell, vesicles are dynamically moving from the rough endoplasmic reticulum to the Golgi apparatus and from the Golgi apparatus to the plasma membrane, for the delivery of new molecular components of the extracellular matrix.

Concerning possible signs of immunological or any other adverse reactions in the connective tissue, inflammatory cells and/or granulomas were never detected.

DISCUSSION

On the basis of our observations, the filler used in this study is safe; it doesn't show signs of stimulation of the immune system and/or any other adverse reactions. Safety and biocompatibility properties of the used filler are guaranteed by hyaluronic acid produced by genetically modified *Bacillus subtilis* carrying the gene encoding the enzyme hyaluronic acid synthase (deriving from *S. equisimilis* genome) (6), and furtherly enhanced by the combined use of PEG. The advantageous properties of PEG, such as its hydrophilicity, non-toxicity and non-immunogenicity, are transferred to the combined filler here used through PEGylation, acting with a supplementary protective action towards the risk of immunological adverse reactions (14). Furthermore, if we consider that our observations in these subjects were made eight months after the injection of the filler, it means that this material was for the most part retained in the hypodermal tissue, with a very low solubilization and a very limited diffusion outside this site.

The injected filler appears harmoniously integrated with the structures inside the connective tissue, as collagen fibres, blood and lymphatic vessels, glands and nerves. Thanks to PEGylation, the filler used presented excellent rheological properties, such as cohesivity, viscoelasticity and plasticity, with an optimized adaptation to the receiving anatomic area

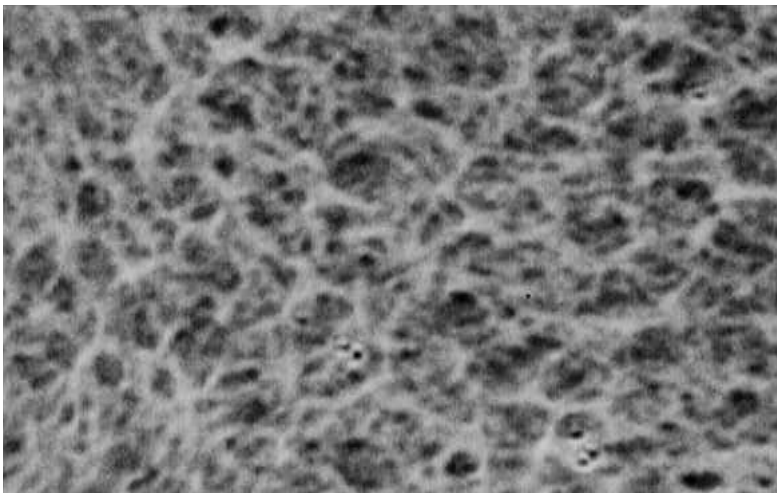

Fig. 3. *Interference light microscopy imaging at vey high magnification. Organization of the molecular complex of the PEGylated filler stained by Alcian blue. 3D network of variable mesh size with knots representing the linking sites between the polymers. Scale bar: 5 μm.*

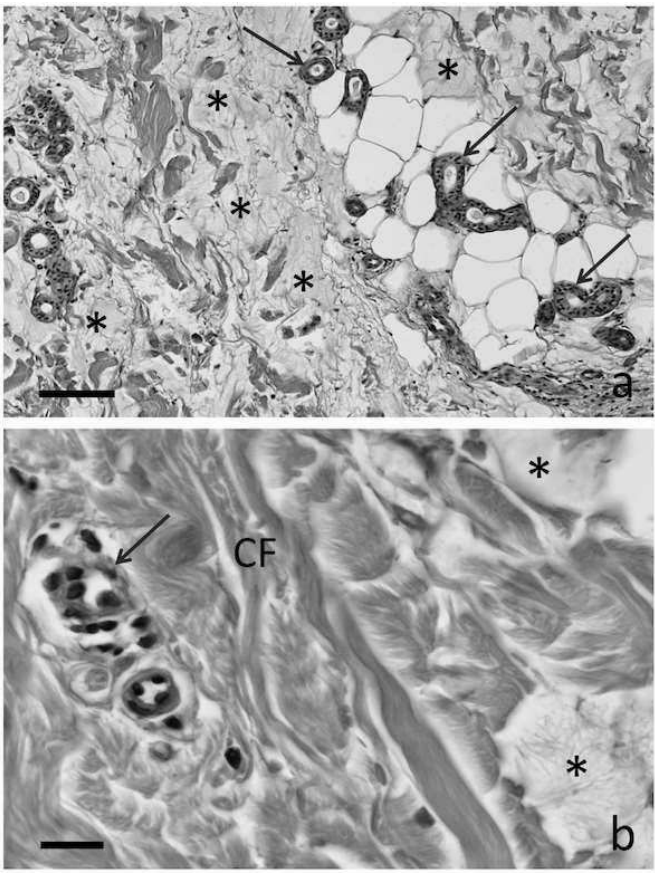

Fig. 4. *Hematoxylin and Eosin stained sagittal section of hypodermis.* ***a)*** *The filler (light blue, asterisks) is perfectly integrated in the connective tissue, among collagen fibres (**red**), adipose tissue (**white**) and some ducts of sweat glands (**arrows**). Scale bar: 100 μm. b) Hypodermal extracellular matrix. Collagen fibres (**CF, red**), differently oriented, appear in close relationship with the filler (**light blue, asterisks**) without any specific encapsulation. On the left: a small excretory duct (**arrow**) and a small arteriole. Scale bar: 20 μm.* ***a)*** *and* ***b)*** *Immunoreactions and/or related cells were never detectable.*

and, at the same time, it maintained the desired shape for a long lasting esthetical correction. In the present study, this was well demonstrated by the limited diffusion of the filler outside the injection site, and the limited dislocation of collagen fibres, glands and other resident structures inside subcutaneous and dermal connective tissue. The desired volume appears maintained without any non-physiological compression to surrounding tissues.

The filler not only gives volume and support to the connective tissue as injected substance, but, due to the very high content of polar groups along the molecular structure, it has also a positive associated effect to link an extraordinary quantity of water molecules (15). In this way, a high hydration of the extracellular matrix of the connective tissue is increased and maintained, thus enhancing extracellular matrix permeability and the diffusion of nutrients from blood vessels to the whole skin as an organ, epidermis included, in a renewed young homeostatic balance.

Findings by electron microscopy have shown in fibroblasts some ultrastructural features of the nucleus and cytoplasmic organelles (rough endoplasmic reticulum with dilated cisternae and an extended Golgi apparatus), which are supporting a functional stimulation to a renewed activity of proteins and glycoproteins synthesis, with following release into the extracellular matrix. In particular, the material inside dilated cisternae could realistically represents procollagen (16), the molecular precursor of collagen and other proteins, which are dynamically transported to the Golgi apparatus where they are glycosilated. Procollagen molecules, through vesicles originated in the Golgi apparatus, are delivered as molecules of tropocollagen to the extracellular matrix for a new collagen fibrillogenesis. Other vesicles from the Golgi apparatus are delivering other glycoproteins and proteoglycanes for the enrichment of the ground substance. These features support a renewed activity of fibroblasts for an active regeneration of the extracellular components of the connective tissue (collagen, glycoproteins, glycosaminoglycans). Thus, the new production of molecular components of the extracellular matrix constitutes a supplementary positive effect. This effect, together with the high hydration grade of the extracellular

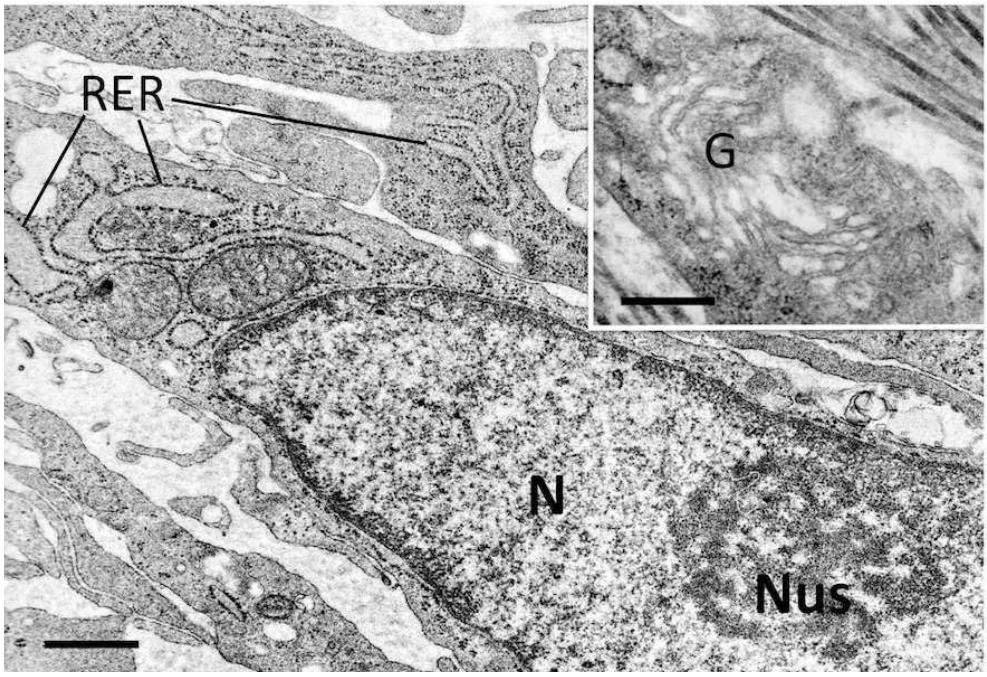

Fig. 5. *Electron microscopy imaging. Fibroblast with highly euchromatic nucleus (**N**), nucleolus (**Nus**) and a high cytoplasmic content of rough endoplasmic reticulum (**RER**) with dilated cisternae rich in ribosomes. Scale bar: 500 nm. In the inset (**top-right**): Golgi apparatus (**G**) presenting dilated membranes and associated vesicles. Scale bar: 500 nm.*

matrix, is representing a natural additional filling action, further enhancing aesthetically appreciable results and functionally synergy with the action of the injected filler.

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