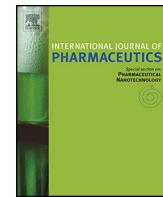




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A new highly viscoelastic hyaluronic acid gel: rheological properties, biocompatibility and clinical investigation in esthetic and restorative surgery

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ABSTRACT

Nowadays there is an increased demand for safe and effective volume enhancing fillers to achieve soft tissue augmentation in order to overcome tissue defects and aging-associated skin changes. In the present study we characterized the rheological and biological properties of Variofill®, a new highly viscoelastic hyaluronic acid gel, by investigating the local effects following subcutaneous implantation in the rat to detect the host-tissue reactions and biodegradation over 18 months. We also investigated, for the first time, the application of Variofill® in esthetic and restorative surgery in two medical case reports. In the first case report we successfully performed Variofill® treatment to improve facial scars in a patient previously involved in a car crash. In the second case report we carried out a novel procedure involving a high-dose (1000 ml) injection of Variofill® into the dermis and subcutis of the abdominal quadrants in order to allow a classic reconstructive procedure of the abdominal wall in a patient presenting a wide incisional hernia.

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1. Introduction

In the era of increased life expectancy (Iannitti and Palmieri, 2011), people are becoming more conscious of their looks (Honigman and Castle, 2006). Furthermore, facial soft tissue deformities and aging-associated skin changes affect psychosocial interactions (John and Price, 2009). Therefore, there is an increased demand for safe and compatible volume enhancing fillers to achieve soft tissue augmentation in order to overcome tissue defects. Filler-based procedures are highly demanded since they are not invasive and do not require downtime (Klein, 2006). Since the earliest experiments with filling materials in the late 1800, physicians have been looking for an ideal injectable biomaterial (Klein, 2006). Injectable materials for soft tissue augmentation have experienced an astounding growth in the US market since the introduction of bovine collagen-based dermal fillers during the 1980s (Brandt and Cazzaniga, 2008). Nowadays, many solutions for volume enhancement are available for the patient and they

include fat transfer, silicone implants, and the use of injectable non-resorbable products such as silicone, polyalkylimide, polyacrylamide gels and resorbable products such as hyaluronic acid (HA) gels (Heden et al., 2009). The ideal filler should be non-allergenic, non-carcinogenic, adverse effect-free and not associated with migration and excessive inflammatory response following its application (John and Price, 2009). Furthermore, filler materials should have a long-term effect and slow degradation in the body. HA, a polysaccharide of the extracellular matrix, possesses structural, rheological, physiological and biological functions in the body, and displays inhibitory effects against a range of bacteria and fungal species as well as antiviral activity (Ardizzone et al., 2011; Cermelli et al., 2011; Leach and Schmidt, 2004). It is a linear and anionic polymer which consists of two modified sugars, D-glucuronic acid and N-acetyl-D-glucosamine (Collins and Birkinshaw, 2008). Native HA has the limitation to be rapidly degraded, residing for just 1–2 days within the tissue (Tezel and Fredrickson, 2008). For this reason, use of chemically cross-linked form of HA is essential to ensure a longer residence time of the compound within the soft tissue (Tezel and Fredrickson, 2008). The cross-linking process is also essential to improve the mechanical properties and control the degradation rate of HA *in vivo*. Previous studies had shown that it can be cross-linked using polyfunctional

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reagents such as bis-epoxides, carbodiimides, and divinyl sulfone (DVS) (Laurent et al., 1994; Kuo et al., 1991; Larsen et al., 1993). In particular, the biocompatibility of cross-linked HA with DVS is well established (Hahn et al., 2007; Oh et al., 2008). DVS attacks HA hydroxyl groups creating a network of HA chains (Campoccia et al., 1998). Furthermore, DVS promptly reacts with HA in aqueous alkaline solutions at room temperature, thereby providing cross-linked HA gels (Balazs and Leschiner, 1986). These gels, once injected into the skin, provide a more complex chemical structure and more robust physical barrier to the enzymatic and free radical degradation of soft tissue fillers (Tezel and Fredrickson, 2008). Cross-linked HA also finds application in many different medical fields including esthetic medicine (Iannitti et al., 2011a), wound healing (Lapčík et al., 1998) and drug delivery (Marusza et al., 2012), and is extensively used for treatment of osteoarthritis (Iannitti et al., 2011b). For several years, HA-based fillers have become the most successful response to the current massive demand for non-surgical soft tissue augmentation. Intra-dermal injections of HA fillers are performed to fill wrinkles and augment the volume of soft tissues such as lips, nasolabial folds and facial wrinkles (Brown and Jones, 2005). Moreover, chemically cross-linked HA has been used as a soft tissue filler for facial rejuvenation, allowing to improve skin elasticity and tautness and enhance rehydration due to its elastic properties that depend on HA molecular weight, concentration and cross-linking process/degree (Bogdan Allemann and Baumann, 2008).

Variofill® (Adoderm GmbH, Langenfeld, Germany), a novel DVS cross-linked HA, has recently come onto the market. A previous study performed in our laboratories shows the efficacy of this product in improving knee articular cartilage degeneration in a

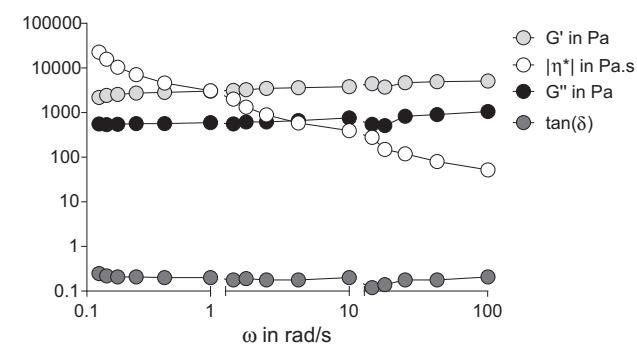


Fig. 1. Frequency (ω) dependence of the viscoelastic properties (elastic and viscous moduli [G' and G'']) of Variofill®.

preclinical model of knee osteoarthritis (Iannitti et al., 2013a). Furthermore, previous evidence from our clinic shows that Variofill® is effective and safe when used for treatment of knee osteoarthritis (Iannitti et al., 2012; Palmieri et al., 2013), lower-leg telangiectasia (Iannitti et al., 2013b) and premature ejaculation (Littara et al., 2013). However, there is no evidence of its efficacy and safety in esthetic and restorative surgery. Therefore we characterized the rheological properties and biocompatibility of Variofill® by investigating its local effects following subcutaneous implantation in the rat to detect the host-tissue reactions and biodegradation over 18 months. Then we translated these findings into the clinical setting using Variofill® for the first time in two patients seeking esthetic and restorative surgical procedures.

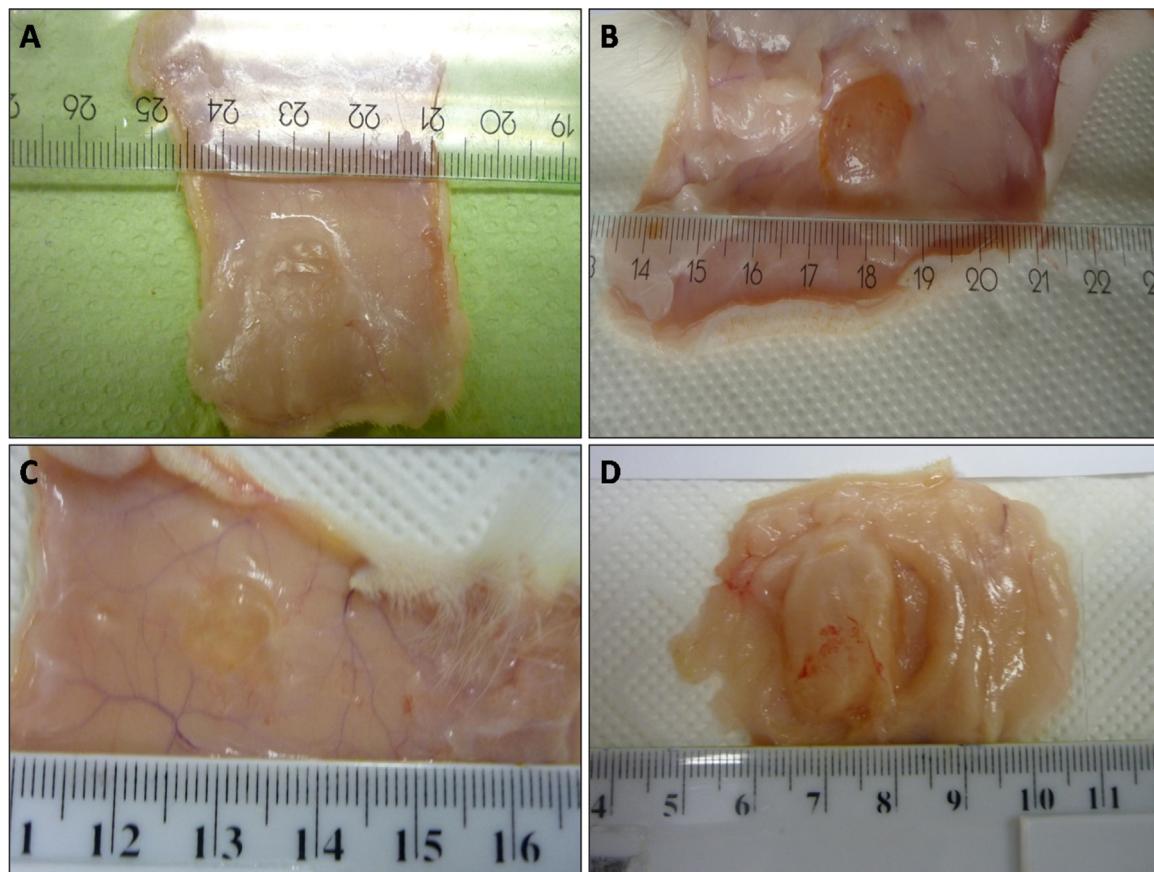


Fig. 2. Gel depot macroscopic appearance. (A) Test group rat (time post-injection = 15 months). (B) Satellite group rat (time post-injection = 15 months). (C) Test group rat (time post-injection = 18 months). (D) Satellite group rat (time post-injection = 18 months).

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in accordance with DIN EN ISO 10993-2:2006. Animal studies were approved by the veterinary and food control office of the Federal State of Mecklenburg-Vorpommern (LVL M-V/TSD/7221.3-2.1). The two patients involved in this investigation signed the informed consent and the procedures were performed in accordance with the Declaration of Helsinki. Institutional Review Board (Poliambulatorio del Secondo Parere, Modena, Italy) approval was also obtained for clinical investigations.

2.2. Preparation of cross-linked HA hydrogel

HA solution of 3.3 wt% concentration was prepared by dissolving 66 g of sodium hyaluronate (1×10^6 Da, Kräber GmbH & Co., Ellerbek, Germany) in 2000 ml of 0.05 mol/l NaOH at room temperature. After complete dissolution, DVS ($\geq 98\%$; Sigma-Aldrich, Germany) was added to the HA solution for the cross-linking reaction with HA hydroxyl groups. A large HA gel mass was obtained and was broken into gel particles ($\sim 150 \mu$; Monophasic Particle Technology [MPT]). HA gel was then washed to remove any unreacted cross-linker reagent. Finally, syringes were filled up with HA gel particles.

3. Experimental studies

3.1. Small amplitude oscillatory shear

The viscoelastic properties of HA gel can be evaluated by rheological testing methods. Rheology builds on the correlation of deformation of a material and the applied stresses and deals in particular with flows (Barnes et al., 1989). Small amplitude oscillatory shear (SAOS) is a rheological method to determine the viscoelastic properties of a material (Macosko, 1994). Rheologically, the viscoelastic characteristics of HA gel can be described by estimating the elastic modulus, represented by the symbol G' (G prime) and the viscous modulus, represented by the symbol G'' (G double prime) using a rheometer (Falcone and Berg, 2009). The degree of modification, the amount of cross-linked and uncross-linked HA and the degree of hydration affect the rheological properties of HA gel (Kablik et al., 2009). The ratio between the viscous modulus and the elastic modulus corresponds to the loss tangent $\tan \delta$ ($\tan \delta = G''/G'$) (Falcone and Berg, 2009). If the $\tan \delta$ is greater than 1, the material is predominantly viscous, and if it is smaller than 1, the material is predominantly elastic (Segura et al., 2012). HA concentration, the degree of HA cross-linking, the amount of uncross-linked HA and the manufacturing process, all contribute to the resulting gel hardness, measured by G' . In our study rheological measurement was performed using a HAAKE RheoStress 1 rheometer (Thermo Fischer Scientific, Process Instruments, Karlsruhe, Germany). Variofill® rheological properties were characterized by SAOS regime, using a plate-and-plate geometry of 35 mm and a gap of 1 mm at 20 °C.

3.2. Implantation test

The implantation test was performed according to DIN EN ISO 10993-6: 2009. The test was applied for the assessment of local effects of the test material, Variofill®, injected subcutaneously into the rat dorsum and compared to control material consisting in 0.3% agarose (NEEO Ultra-Quality, Carl-Roth, Darmstadt, Germany). This test is routinely applied for the assessment of local effects of an

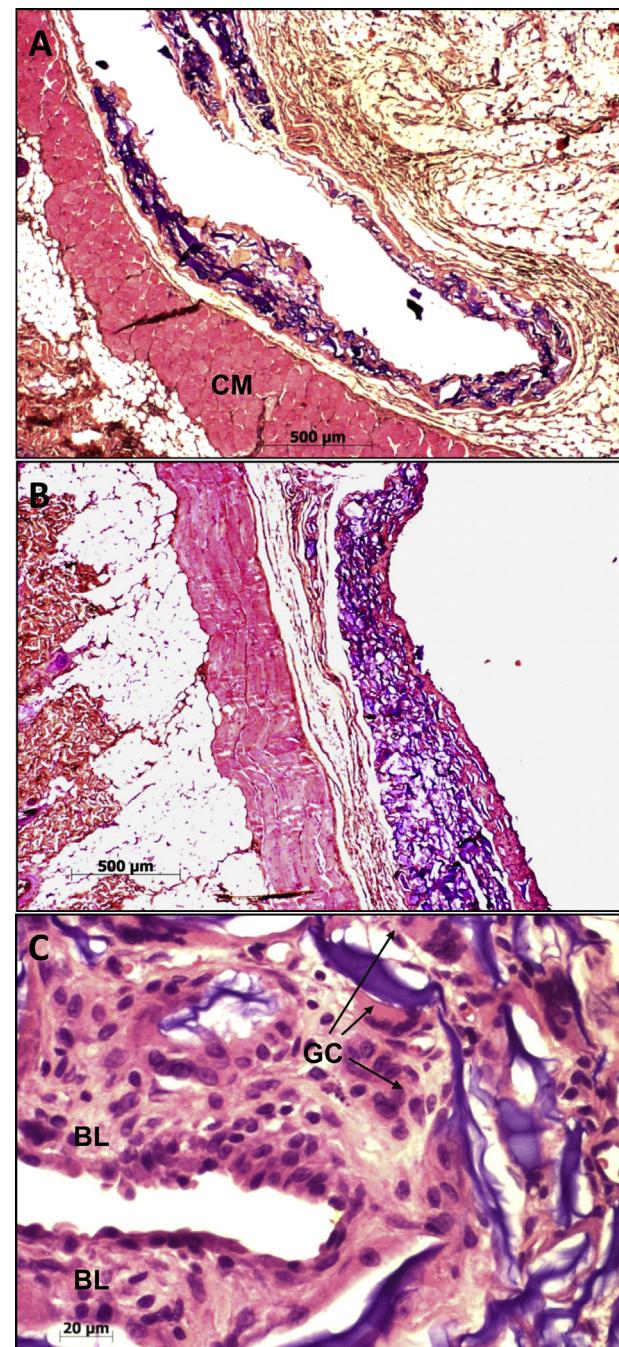


Fig. 3. (A) Test group rat (time post-injection = 6 months). Gel depot over the cutaneous muscle and narrow capsule formation. Subcutaneous fat tissue between cutaneous muscle and cutis is visible on the left side. Slight fibrosis is visible. (B) Satellite group rat (time post-injection = 6 months). Gel depot capsule with gel islands over the cutaneous muscle. Slight to moderate fibrosis is visible. (C) Satellite group rat (time post-injection = 6 months). Gel depot capsule with gel islands. CM = cutaneous muscle; BL = border layer; GC = giant cells. Hematoxylin and eosin stain.

implant on animal tissue at macroscopic and microscopic level (DIN EN ISO 10993-6: 2009; Martin et al., 1990; Rusiecki et al., 2004).

3.3. Animals and study design

A total of 30 adult female Wistar rats (Charles River Laboratories, Germany; 176–200 g), aged 7 weeks, were used in this investigation. Animals were housed at 18 ± 3 °C and $60 \pm 20\%$ humidity. They

had ad libitum access to food and water. Rats were maintained on a 12 h light/dark cycle.

Rats were divided into 3 groups: group one ($n=10$) received a subcutaneous injection of 0.25 ml Variofill® (test group); group 2 ($n=10$) received a subcutaneous injection of 1 ml Variofill® (satellite group); group 3 ($n=10$) received 0.25 ml of 0.3% agarose and served as control. All the injections were performed under sterile conditions and by the same operator throughout the study. Control solution was prepared dissolving agarose in Phosphate Buffered Saline (PBS) and was chosen since it is non-toxic and it has a consistency similar to Variofill®. Following Variofill® injection, two animals from each group were euthanized with an intraperitoneal injection of diethyl ether (Carl Roth, Darmstadt, Germany) at 6, 9, 12, 15, and 18 months. Samples of the skin surrounding the site of injection were collected for macroscopic and microscopic assessment. For this purpose, the lateral implantation areas, as well as some untreated dermis sites, were excised starting from the deep muscular layer up to the skin.

3.4. Gross pathology and histology

Gross pathological observation of the implantation area, photographed after the injection of materials and before euthanizing the rats, was performed according to Oh et al. (Oh et al., 2008). Animals were euthanized with 0.6 ml of 10% ketamin (WDT, Garbsen, Germany) and 0.2 ml of 2% xylazin (Serumwerk Bernburg AG, Bernburg, Germany). For histological investigation, 4% neutrally buffered formalin-fixed and paraffin-embedded tissue sections were stained with hematoxylin (Merck, Darmstadt, Germany) and eosin (Fa. Applichem, Darmstadt, Germany) according to our previous study (Iannitti et al., 2013a).

Macroscopic findings (Axio Scope.A1, Carl Zeiss GmbH Microlmaging, Göttingen, Germany) at implantation sites and untreated skin sites were analyzed. The small and big diameter of gel depot

areas were measured. Extent of fibrosis was classified as minimal, minimal to slight, slight, slight to moderate and moderate.

4. Clinical study

4.1. Patients

The two patients involved in this investigation were treated at the "Poliambulatorio del Secondo Parere" (Modena, Italy). The first patient presented traumatic scars disfiguring his face profile. The second patient presented a severe abdominal incisional hernia directly overlapping a small parietal peritoneum. Both cases underwent a medical procedure based on Variofill®.

4.2. Histological analysis

Round skin grafts (2 mm) from the area injected with HA gel were taken from both patients using a dermatome. Then samples were fixed in a 10% formalin solution (Sigma-Aldrich, Milan, Italy) for 48 h at room temperature. Paraffin tissue processing was performed according to the following steps: (1) 70% ethanol (Sigma-Aldrich, Milan, Italy) for 20 min; (2) 95% ethanol for 20 min (twice); (3) 100% ethanol for 20 min (twice); (4) xylene (Sigma-Aldrich, Milan, Italy) for 1 h; (5) paraffin (65 °C; Sigma-Aldrich, Milan, Italy) for 1 h; (6) paraffin (65 °C) with vacuum for 1 h. Blocks to be sectioned were placed face down on an ice block for 10 min and sectioned at the freezing microtome. Slides were deparaffinized and processed according to the following protocol: xylene for 10 min, 100% ethanol for 6 min, 95% ethanol for 2 min, 70% ethanol for 2 min, 50% ethanol for 2 min and distilled water for 2 min. Then they were stained in 1% alcian blue (Alcian Blue 8GX; Sigma-Aldrich, Milan, Italy) solution dissolved in 0.8% acetic acid for 10 min and washed in running tap water for 10 min. Finally, they were counterstained in nuclear fast red solution (Sigma-Aldrich, Milan, Italy) for 5 min. Slides were dehydrated

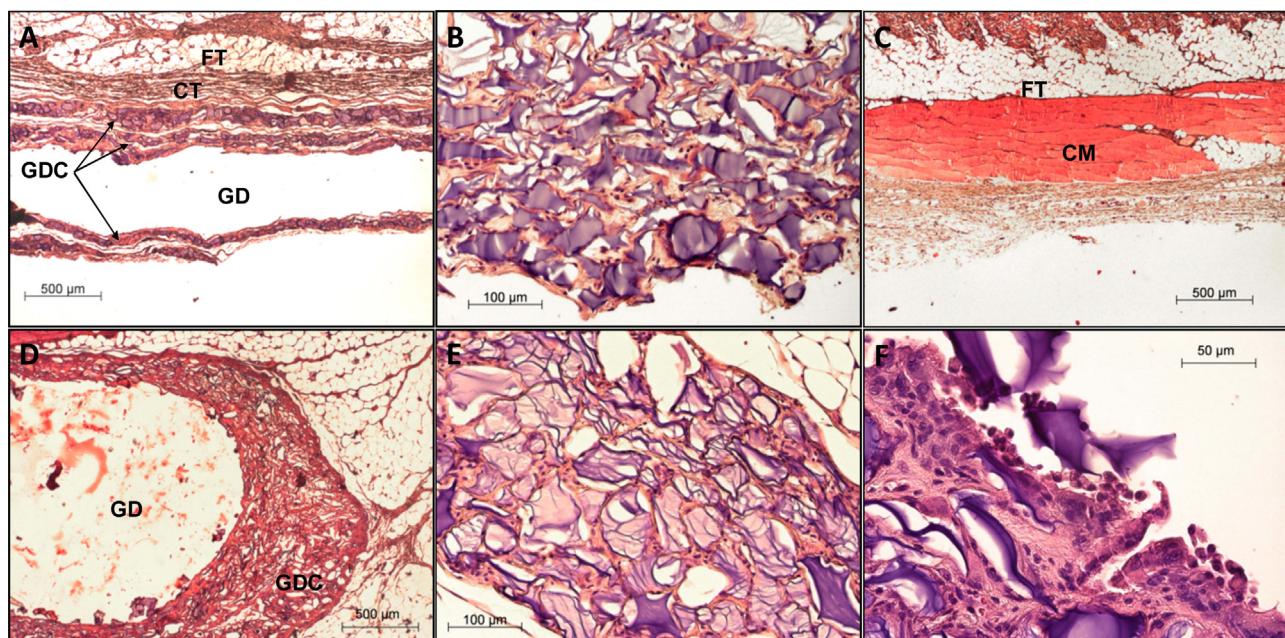


Fig. 4. (A) Test group rat (time post-injection = 9 months). Gel depot and gel depot capsule. HA gel is well integrated within the dermal tissue. (B) Test group rat (time post-injection = 9 months). Gel depot capsule. Slight to moderate fibrosis and slight infiltration of inflammatory cells are visible. (C) Test group rat (time post-injection = 9 months). Normal untreated skin area. Normal connective tissue can be seen underneath the cutaneous muscle. (D) Test group rat (time post-injection = 9 months). Gel depot with thick part of gel depot capsule. Moderate fibrosis is visible. (E) Satellite group rat (time post-injection = 9 months). Section of gel depot capsule. Slight fibrosis and slight infiltration of inflammatory cells are visible. (F) Satellite group rat (time post-injection = 9 months). Gel depot capsule. To some degree, dense infiltration of inflammatory cells (macrophages and giant cells) is visible in the border layer. GD = gel depot; GDC = gel depot capsule; FT = fat tissue; CT = connective tissue; CM = cutaneous muscle. Hematoxylin and eosin stain.

and mounted with resinous mounting medium (Poly-mount, Polysciences Inc., Warrington, US).

5. Results

5.1. Rheological properties

Viscoelastic properties of Variofill® were measured using oscillatory shear rheology. Elastic and viscous properties of Variofill® at different frequencies are shown in Fig. 1. Variofill® showed elastic modulus (G') of 2850 Pa, viscous modulus (G'') of 567 Pa and $\tan(\delta)$ of 0.20 at the frequency of 0.63 rad/s.

5.2. Macroscopic findings

Gel depot at 15 and 18 month follow-up in the test and satellite groups is shown in Fig. 2A–D. No gel depot was seen in the control group. In all groups, no abnormality was detected in the untreated skin sites surrounding the implantation area throughout the study. Example of normal untreated skin area from a test group rat at 9 month follow-up is shown in Fig. 4C.

5.3. Histological analysis

Variofil® depots decreased during the study due to biodegradation. The gel depots exhibited variable thickness from rat to rat and were enclosed by a capsule layer (Figs. 4A, D, 5A–B, 6A–D). There were only a few small basophil gel parts close to the capsule layer. This layer consists of a framework of fibrous septa that differ in density and thickness and enclose small gel islands (Figs. 3B–C, 6B, D, 7B–C and 8A). There is a fibrous borderline layer (thickness differs from slide to slide) close to the inner big gel depot. The outer side of the capsule layer is surrounded by connective tissue with different thickness. The total extent of fibrosis is the confluence and fusion of the outer connective tissue in gross trabecular strands of the inner thin collagen borderline layer and the mesenchymal

reaction surrounding the persistent gel depots. The extent of fibrosis and infiltration of inflammatory cells of test group and satellite group animals, after 6 (Fig. 3A–B), 9 (Figs. 4B, D–F and 5A–B), 12 (Fig. 6B–D), 15 (Fig. 7B–C) and 18 (Fig. 8A–C) months post-injection, is summarized in Table 1.

Test group. In the low-dose HA group, histological analysis at 6 months showed multiple HA metachromatic vacuoles in the dermal stroma over the cutaneous muscle (Fig. 3A). At 9 month follow-up, HA gel was well integrated into the dermal tissue (Fig. 4A). In the surrounding areas, slight to moderate fibrosis within an intact collagen network and slight infiltration of inflammatory cells were observed (Fig. 4B). HA gel deposits across the muscle fibers, without damage to the muscle morphology, were observed at 12 months (Fig. 6B). In these deep foci, moderate confluence of the cross-linked HA into larger pools, with slight or moderate collagen fibers surrounding these areas, was also detected (Fig. 6B). At 15 months, a deep subcutaneous pool of HA gel between muscle and subcutaneous adnexa was still present suggesting that absorption of the biomaterial was ongoing, but further HA gel remnants among clusters of fatty cells were still persisting (Fig. 7A). Finally, at 18 months, HA gel was partially biodegraded and was pooled into metachromatic vacuoles surrounded by fibrous strands and only slight infiltration of inflammatory cells (Fig. 8A).

Satellite group. In the high-dose HA group, histological analysis at 6 months showed that the biomaterial was surrounded by moderate collagen strands with several fibroblasts actively secreting procollagen bands (Fig. 3C). At 9 month follow-up, HA gel was visible together with a slight inhomogeneous fibrotic lining in the dermis and muscular fascia (Fig. 5A), with slight or absent infiltration of inflammatory cells (Figs. 4E and 5B). At 12 months, HA strands formed layers between deep dermis and subcutis and slight fibrosis and infiltration of inflammatory cells were visible (Fig. 6D). At 15 months, HA gel remnants were pooled into large diameter (50–60 μm) vacuoles surrounded by a moderate capsular reaction outside of the initially injected HA gel volume (Fig. 7B). Moreover, HA gel remnants were intermingled with mature fibroblasts

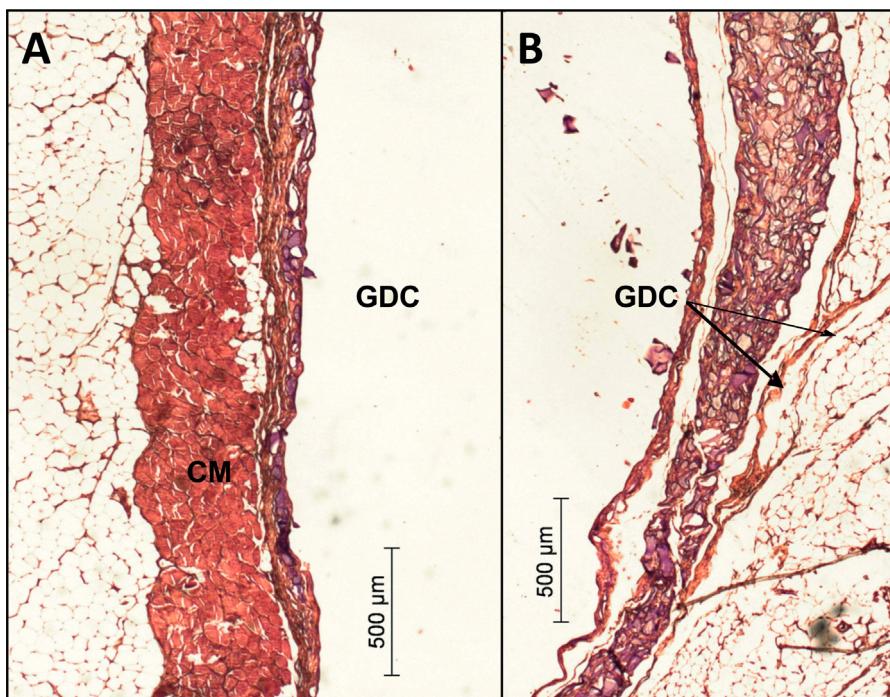


Fig. 5. Satellite group rat (time post-injection = 9 months). (A) Capsule is thin in the dermal space and very thin under the epidermis. Slight fibrosis is visible. (B) No inflammatory reaction and scanty collagen strands are visible across the reticular stroma. CM = cutaneous muscle. GDC = gel depot capsule. Hematoxylin and eosin stain.

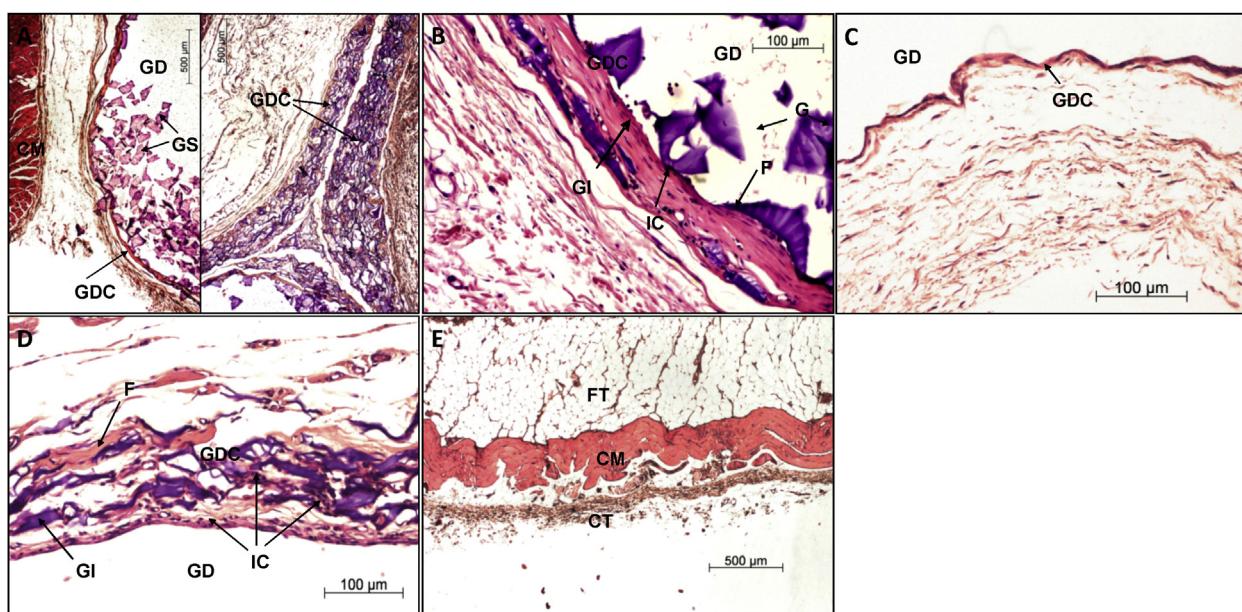


Fig. 6. (A) Test group rat (time post-injection = 12 months). Gel depot, surrounded by a very thin capsule and two thin to moderately thick layers, is differently layered in the dermis. Narrow gap between the layers is visible. (B) Test group rat (time post-injection = 12 months). Section of a very thin gel depot capsule showing slight fibrosis and minimal infiltration of inflammatory cells. (C) Test group rat (time post-injection = 12 months). Section of very thin gel depot capsule. Minimal fibrosis is visible. (D) Satellite group rat (time post-injection = 12 months). Section of gel depot capsule showing slight fibrosis and slight infiltration of inflammatory cells. (E) Control group rat (time post-injection = 12 months). CM = cutaneous muscle; GD = gel depot; GDC = gel depot capsule; GS = gel sheets; F = fibrosis; IC = inflammatory cells; GI = gel island; G = gel; FT = fat tissue; CT = connective tissue.

Hematoxylin and eosin stain.

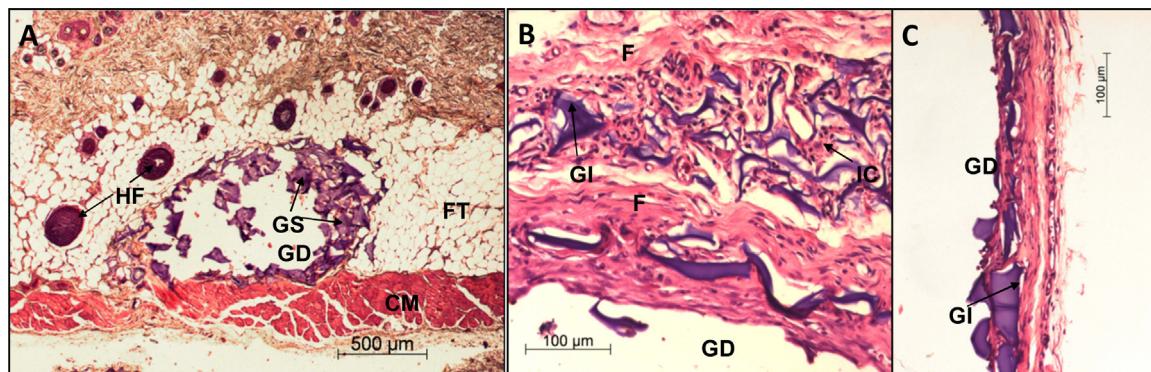


Fig. 7. (A) Test group rat (time post-injection = 15 months). Small gel depot in subcutis above cutaneous muscle with very thin capsule within gel depot sheets. (B) Satellite group rat (time post-injection = 15 months). Section of gel depot. Moderate fibrosis and moderate infiltration of inflammatory cells are visible. (C) Satellite group rat (time post-injection = 15 months). Slight fibrosis and minimal infiltration of inflammatory cells can be seen. GD = gel depot; CM = cutaneous muscle; GS = gel sheets; FT = fat tissue; HF = hair follicle; F = fibrosis; IC = inflammatory cells; GI = gel islands.

Hematoxylin and eosin stain.

Table 1

Time course of average fibrosis and infiltration of inflammatory cells in test and satellite group animals after subcutaneous injection of Variofill®.

| Time | Group | Fibrosis | Inflammation |
|-----------|-----------------|---------------------|--------------------|
| 6 months | Test group | Slight | Minimal |
| | Satellite group | Slight to moderate | Moderate |
| 9 months | Test group | Slight to moderate | Slight |
| | Satellite group | Slight to moderate | Slight to moderate |
| 12 months | Test group | Minimal to moderate | Minimal to slight |
| | Satellite group | Slight | Slight |
| 15 months | Test group | Slight to moderate | Minimal to slight |
| | Satellite group | Minimal to moderate | Slight to moderate |
| 18 months | Test group | Slight | Minimal to slight |
| | Satellite group | Slight | Minimal to slight |

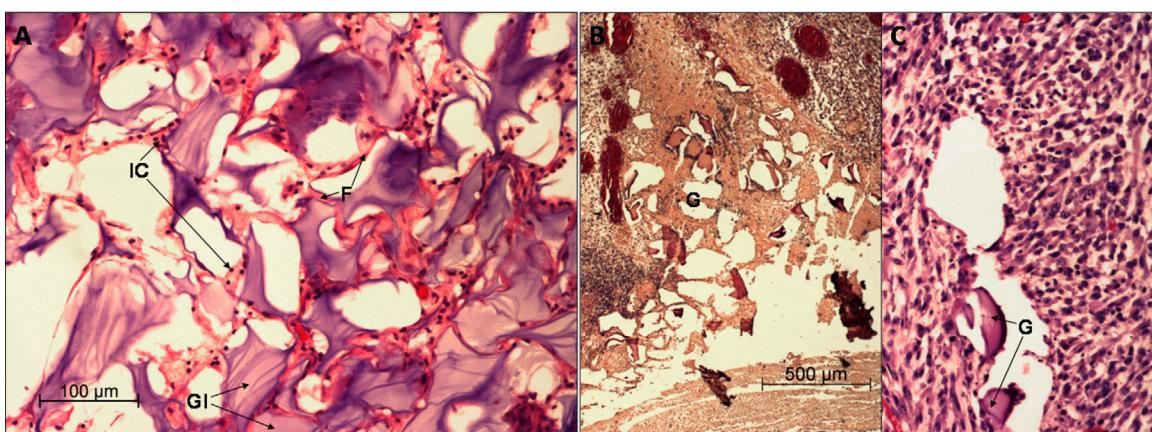


Fig. 8. (A) Test group rat (time post-injection = 18 months). HA gel is pooled into metachromatic vacuoles surrounded by fibrous strands. Slight infiltration of inflammatory cells and slight fibrosis are visible. (B) Satellite group rat (time post-injection = 18 months). Some empty vacuoles, previously filled with HA gel that have now been cleared and digested, can be observed. Moderate inflammatory reaction is visible. (C) Satellite group rat (time post-injection = 18 months). Area of moderate inflammatory reaction with mixed leukocyte infiltration and remodeling of the normal skin architecture. IC = inflammatory cells; F = fibrosis; GI = gel islands; G = gel. Hematoxylin and eosin stain.

(Fig. 7B). At 18 month follow-up, HA gel was partially biodegraded and some empty vacuoles, previously filled with HA gel and already cleared and digested, could be seen (Fig. 8B). A moderate inflammatory reaction following high-dose administration could be observed at 18 months (Fig. 8B). Mixed leukocyte infiltration and remodeling of the normal skin architecture could also be observed in this group (Fig. 8C).

Control group. In control group rats the histology of the skin was normal in appearance and no gel depot was detected throughout the study. Histology of a control rat skin at 12 month follow-up is shown in Fig. 6E.

6. Clinical investigation

6.1. Case 1

A 24-year-young man arrived at our clinic with facial scars following a car crash (Fig. 9A). After disinfecting and trimming the injured necrotic skin, dermis and subcutis, 2 ml of Variofill® were injected using a 31G 5 mm long mesotherapy needle (Fig. 9B–C) to lift up the surrounding injured and depressed scar areas, also performing the injections around the injured subcutis. At 3 month follow-up, a remarkable improvement in the eyelid and facial scars

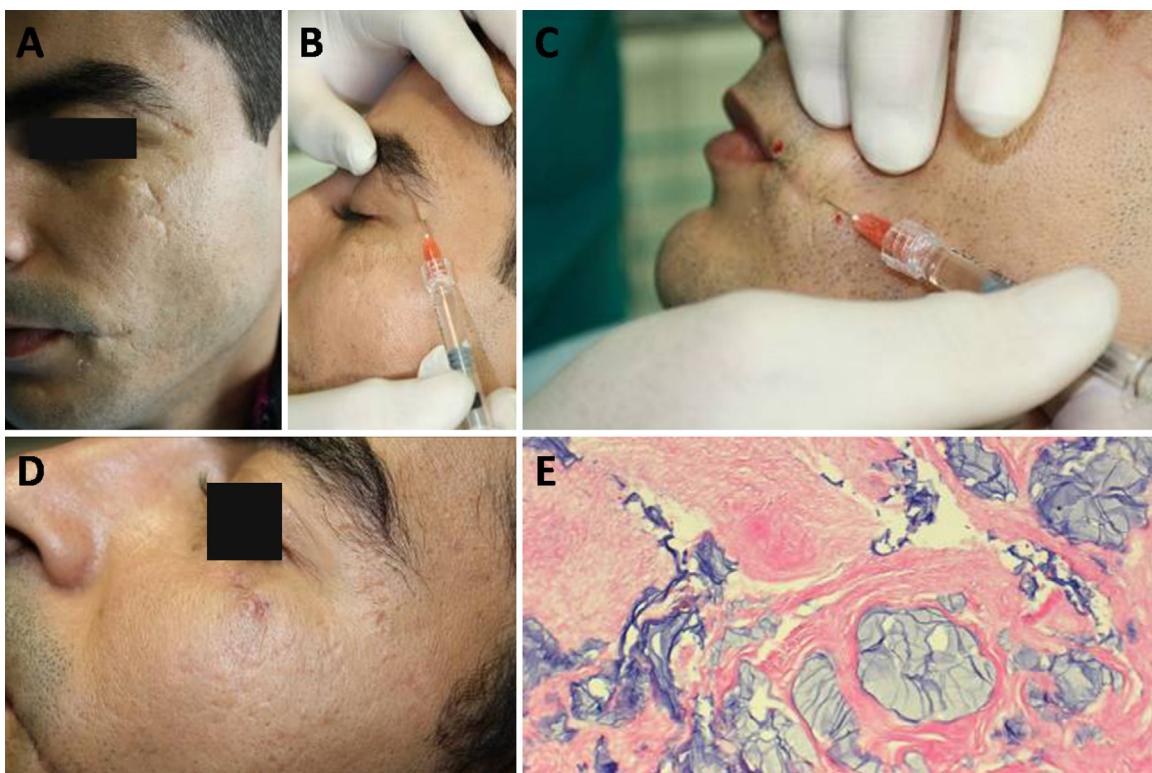


Fig. 9. (A) Patient presenting facial scars following a car crash. (B) HA gel injection in the eyelid. (C) HA gel injection in the perioral area near to the scar. (D) Evident improvement in the eyelid and facial scars at 3 month follow-up. (E) HA gel is well integrated into the dermis without inflammatory reaction at 3 month follow-up. Alcian blue stain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

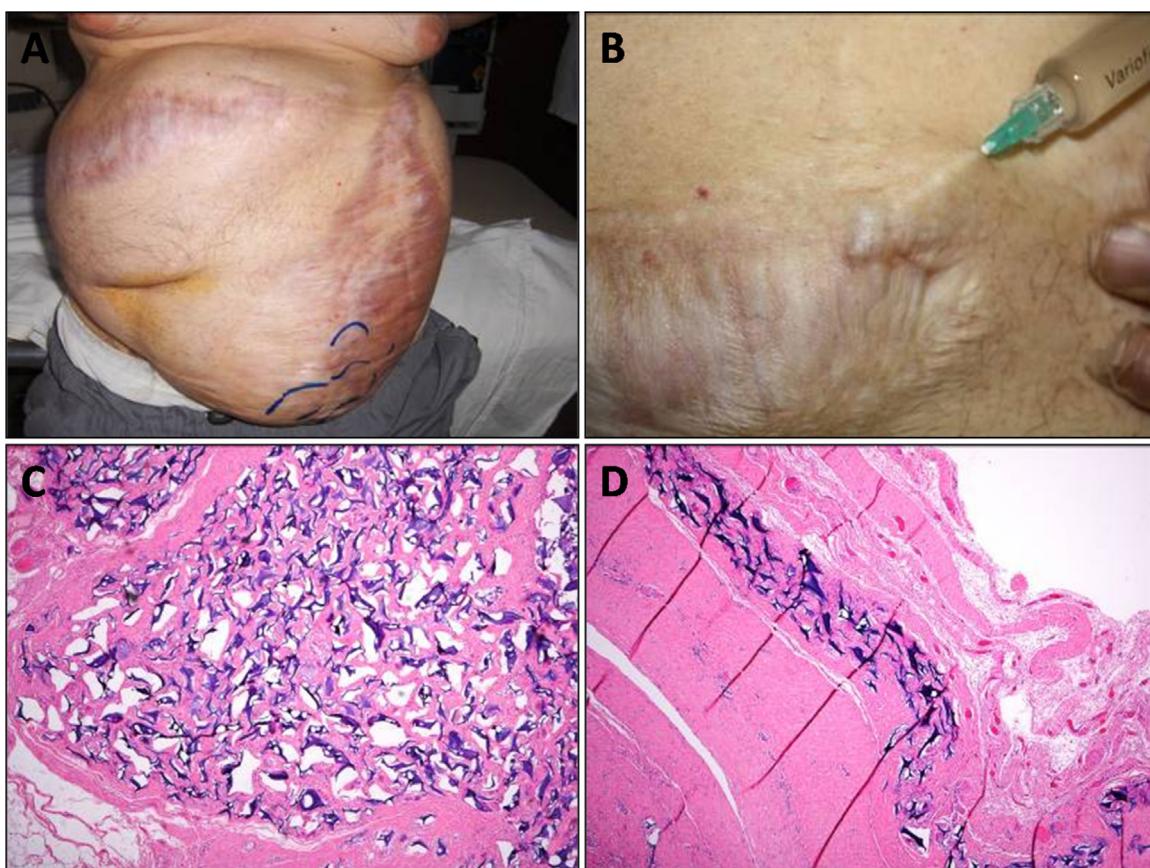


Fig. 10. (A) Patient with an extremely thinned skin covering an outstanding laparocele. (B) HA gel injection to enhance the dermis, deep dermis and subcutis thickness. (C) and (D) HA gel (violet) is well integrated into the dermis at 14 month follow-up. Alcian blue stain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

was observed (Fig. 9D). Histological analysis of dermis samples, previously treated with HA, showed that the compound was well integrated without inflammatory reaction (Fig. 9E).

6.2. Case 2

A 62-year-old man arrived at our clinic with an extremely thinned skin covering a wide incisional hernia involving the whole anterior abdominal wall (Fig. 10A). Following local anesthesia with carbocaine 1% (Astra Zeneca, Milan, Italy), the patient was injected with 1000 ml Variofill® using a 25G needle aiming to enhance the dermis, deep dermis and subcutis thickness (Fig. 10B). The outpatient procedure was performed with a 16G cannula introduced through four holes made in the skin of the abdominal quadrants. At the 14 month follow-up, he underwent regular plastic surgery procedure introducing a wide collagen mesh into the peritoneal space to better contain the bowel loops into the abdominal cavity. This procedure was made possible because of the Variofill®-induced enhanced dermis and subcutis thickness. In fact, histological analysis of dermis and subcutis samples at 14 month follow-up showed the filler persistence, sometimes in small, capsulated, inert masses, without remarkable inflammatory cell reaction (Fig. 10C–D).

7. Discussion

7.1. Variofill® displays higher elastic modulus if compared to currently available HA-based fillers

We designed the present investigation to characterize a new highly viscoelastic HA gel, Variofill®. From a rheological

perspective, Variofill® showed an elastic modulus G' equal to 2850 Pa at 0.63 rad/s that is higher than several currently commercially available HA-based fillers (Edsman et al., 2012). A high G' in a HA filler is a predictor of better resistance to skin tension forces (Sundaram et al., 2010). Elasticity represents the gel ability to resist deformation by an applied force. A gel with a higher G' better resists alterations in shape and it is firmer, harder or more elastic if compared to a gel with a lower G' (Stocks et al., 2011). This parameter is determined by HA concentration, the molecular weight of the native HA and the share of non-cross-linked HA found in the end product (Tezel and Fredrickson, 2008). The elastic modulus can also be used to determine the suitability of a HA filler for a specific application (Segura et al., 2012). HA fillers with a greater elastic modulus have a better filling effect and a longer retention time in soft tissue (Weidmann, 2009). This cumulative evidence suggests that Variofill® possesses a high elasticity that can result in a better volumizing effect and long-lasting augmentation in the soft tissue. Furthermore, gels with numerically higher values of G'' are described as being more viscous than gels with a lower G'' (Stocks et al., 2011). Given its rheological profile, when a rejuvenation procedure is performed, Variofill® may be given at a lower dose if compared to several currently available HA-based fillers.

7.2. Variofill® produces minimal to moderate fibrosis and inflammation in the rat model and is partially absorbed within 18 months

In a second set of studies we investigated the local effects of Variofill® after subcutaneous injection in the rat. In the animal model, HA implantation did not produce any abnormalities in

the skin surrounding the implantation site throughout the study. Histological investigation showed that the total extent of fibrosis and inflammatory reaction ranged from minimal to moderate often without major changes in the macroscopic and microscopic appearance throughout the study. After subcutaneous administration, Variofill® triggers an intense fibroblast, stromal and cellular reaction with only minimal to moderate inflammation within 18 months. Both 1 ml and 0.25 ml doses can be detected in the tissue up to 18 months when they are partially absorbed. If compared to the low dose, high-dose HA gel elicits an increased inflammatory response with mixed leukocyte infiltration and normal skin architecture remodeling that are still detectable at 18 month follow-up. While a previous study using different HA hydrogels showed biocompatibility of these compounds up to 24 weeks (Oh et al., 2008), our study extends our understanding of the biological and physiological effects of highly cross-linked HA in the dermis up to 18 months. Our findings suggest that Variofill® has long persistence within the tissue. This characteristic can be of crucial importance when designing a rejuvenation or restorative surgical procedure, thus opening new perspectives on the use of high-density cross-linked HA in the clinical setting.

7.3. Variofill® can be effectively and safely used for esthetic and restorative surgical procedures

After establishing Variofill® biocompatibility in the rat model, we tested this compound for the first time in two patients undergoing esthetic and restorative surgical procedures, respectively. The procedures were completely uneventful and safe. In the first case, histological analysis of dermis and subcutis samples, at 3 month follow-up, revealed that HA gel was well integrated without promoting local inflammation (Fig. 9E) and confirming findings from the animal model. In the second case, we performed for the first time a high volume injection of Variofill® to replace the abdominal thinned prefascial layer. At 14 month follow-up, histological analysis showed enhancement of dermis and subcutis thickness allowing the introduction of a collagen mesh into the peritoneal space. At this time point, HA gel was visible in the form of droplets surrounded by collagen reaction, as observed by histological analysis (Fig. 10C and D).

8. Conclusions

Our rheological study, 18 month implantation test and clinical investigations give positive encouragement to use Variofill® in esthetic and restorative surgery warranting further studies in larger cohorts of patients.

Conflict of interest statement

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Statement of authorship

The authors hereby certify that all work contained in this article is original. The authors claim full responsibility for the contents of the article.

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