Esperienze Dermatologiche 2019 March;21(1):22-6
DOI: 10.23736/S1128-9155.19.00477-1

ORIGINAL ARTICLE

Optimization of the lipofilling procedure with hybrid cooperative complexes of high and low molecular weight hyaluronic acid: preliminary experiments

Ottimizzazione della procedura di lipofilling con complessi ibridi cooperativi di acido ialuronico ad alto e basso peso molecolare: esperienze preliminari

Antonello TATEO [1, 2, 3] *, Giuseppe SOFO [1]

[1]TBClinic Milano Aesthetic Surgery and Medicine, Milan, Italy; [2]Unit of Plastic Surgery, Istituto Auxologico Italiano IRCCS Centro Diagnostico San Carlo-Capitanio, Milan, Italy; [3]Breast Unit, Clinica San Carlo Paderno Dugnano, Milan, Italy

*Corresponding author: Antonello Tateo, TBClinic Milano Aesthetic Surgery and Medicine, Piazza Caiazzo 1, 20124 Milan, Italy.
E-mail: tateoantonello@gmail.com

Abstract - Riassunto

BACKGROUND: Efforts have been made in recent years to standardize the lipofilling procedure in order to improve adipose tissue engraftment at the recipient site in an attempt to achieve a better volumizing result months after surgery. This procedure is, however, still burdened by the resorption of variable amounts of the graft. Recently, a new hyaluronic acid made up of hybrid cooperative complexes (HCCs) has demonstrated an ability to increase the differentiation and proliferation of adipocyte-derived stem cells (ASCs). Based on recent evidence regarding fat grafts and hyaluronic acid, this preliminary experiment was carried out to verify whether the two implant materials may be complementary. The aim of this pilot study was therefore to test a hypothetical complementary approach between lipofilling and hyaluronic acid hybrid cooperative complexes to suggest a possible solution to the problem of graft resorption in the adipose transplantation procedure. This preliminary experiment has confirmed the effectiveness of HCCs on the proliferation of ASCs and the preservation of adipocyte vitality.
METHODS: Thirteen patients were enrolled in this study and four laboratory test were performed.
RESULTS: The sample treated with the closed-circuit washing system was the most concentrated in terms of percentage of adipose tissue (60%) compared to the control (55%). The sample purified with the same system and with the addition of 5% HCCs, although less concentrated (58%) than the sample without additions, was still more concentrated than the control (55%). The sample with 10% HCCs added presented adipocyte concentrations comparable to the control.
CONCLUSIONS: Based on the results obtained, the efficacy of HCCs on the proliferation of ASCs and on the preservation of the vitality of adipocytes is confirmed.

(*Cite this article as:* Tateo A, Sofo G. Optimization of the lipofilling procedure with hybrid cooperative complexes of high and low molecular weight hyaluronic acid: preliminary experiments. Esperienze Dermatol 2019;21:22-6. DOI: 10.23736/S1128-9155.19.00477-1)

KEY WORDS: Hyaluronic acid; Adipocytes; Adipose tissue; Transplantation.

OBIETTIVO: Negli ultimi anni, si è cercato di standardizzare la procedura di lipofilling per migliorare l'attecchimento del tessuto adiposo innestato a livello del sito ricevente, nel tentativo di ottenere un miglior risultato volumizzante a distanza di mesi dall'atto chirurgico. Tuttavia, questa procedura rimane comunque gravata dal riassorbimento di quote variabili dell'innesto. Recentemente, un nuovo acido ialuronico formato da complessi ibridi cooperativi (hybrid cooperative complexes [HCCs]) ha dimostrato la capacità di incrementare la differenziazione e proliferazione delle cellule staminali di derivazione adipocitaria (adipocyte-derived stem cells [ASC]). Sulla base di recenti evidenze sull'innesto di grasso e sull'acido ialuronico, questa esperienza preliminare è stata condotta per verificare se i due materiali da impianto possano essere complementari. Pertanto, lo scopo di questo studio pilota è stato quello di testare un ipotetico approccio complementare tra lipofilling e complessi cooperativi ibridi di acido ialuronico, nel tentativo di offrire una possibile soluzione al problema del riassorbimento dell'innesto nella procedura di trapianto adiposo. Questa esperienza preliminare ha confermato l'efficacia degli HCCs sulla proliferazione delle ASCs e sulla preservazione della vitalità adipocitaria.
METODI: Lo studio ha coinvolto tredici pazienti e sono stati effettuati quattro test di laboratorio.
RISULTATI: Il campione trattato con il sistema di lavaggio a circuito chiuso era il più concentrato in termini di percentuale di tessuto adiposo

(60%) rispetto al gruppo di controllo (55%). Il campione purificato con lo stesso sistema e con l'aggiunta del 5% di HCC, sebbene meno concentrato (58%) rispetto al campione senza aggiunte, era ancora più concentrato rispetto al gruppo di controllo (55%). Il campione con HCC al 10% aggiunto ha presentato concentrazioni di adipociti paragonabili al controllo.
CONCLUSIONI: Sulla base dei risultati ottenuti, l'efficacia degli HCC sulla proliferazione delle ASC e sulla conservazione della vitalità degli adipociti è confermata.

In the constant evolution of scientific research on mini-invasive treatments for facial rejuvenation, attention is focusing on methods that are as physiological and safe as possible, not only regarding the materials used but also in consideration of the aesthetic results obtained.

Autologous fat and hyaluronic acid (HA) are being increasingly used, particularly in the field of the volumetric correction of soft tissues and wrinkles in the face and neck. These elements respond to the need to use substances that are not only biocompatible and naturally derived, but also totally reabsorbable.

Although high performers in terms of corrective capacity and maintenance of volumizing effect, non-absorbable synthetic materials of non-biological origin have proved to have significant limitations over time not only because of their long-term inflammatory and granulomatous reactions but also for their unnatural dynamic effect and the impossibility of modulating aging correction in the long term.[1, 2]

Autologous fat grafting is a mini-invasive surgical procedure that can be performed in an operating room for small operations or in a fully equipped surgery room. As hyaluronic acid implants are commercially available in prefilled syringes, they can be performed in a routine outpatient clinic.

In particular, HA, a fundamental component of the extra-cellular matrix, occupies a prominent place in regenerative medicine, not only because of its ability to retain large amounts of water, while maintaining elasticity and viscosity in the connective tissue, but also because of its ability to stimulate fibroblasts to produce collagen and elastin.[3] More specifically, high molecular weight HA has a structuring effect on the dermal matrix and connective tissue in general, while only low/medium molecular weight HA has the ability to adhere to fibroblastic receptors (CD44) causing them to develop and produce connective tissue.[3-5]

In recent years, adipose tissue, still in a regenerative context, has been broadly re-assessed, after the discovery of a subpopulation of stromal mesenchymal stem cells (ASCs) in its content. It has in fact been widely demonstrated that adipose tissue, resulting from the embryonic mesenchyme, contains a large population of stem cells, capable of self-renewal and differentiation, within its stromal compartment.[6, 7]

Consequently, fat grafting has been used in an effort to standardize the procedure and optimize the cellular engraftment of volumizing adipocytes in parallel with the preservation and transfer of ASCs. Despite this, a number of authors continue to report that the outcome of the procedure is inconsistent both in terms of adipose tissue engraftment and in regenerative terms and point out that there is still much to do to optimize this method.[8]

A recent scientific paper demonstrated the effects of an innovative hybrid molecular structure of HA (hybrid cooperative complexes [HCCs]) on adipose tissue and ASCs. Specifically, the authors of the research tested and compared, in an *in vitro* study, the influence on adipose cell cultures of HA-based gel formulations in free form, in the form of HCCs and in the form of cross-linked HA. The conclusion of the paper shows that HCCs enhance the differentiation of ASCs, while preserving the structure and vitality of the adipocytes. It has been deduced, therefore, that HCCs have effects on the differentiation of hypodermis fat cells, counteracting the effect of the tissue resorption typical of aging.[9]

The conclusions of this paper concern, however, the effect of a pure HCCs graft when injected as a volumizing and bioregenerating filler. Our idea, instead, was to exploit and evaluate the activity of this molecular structure of HA on fat harvested for grafting purposes, as happens in the lipofilling technique.

On the basis of these observations and hence of our hypothesis, we set ourselves the aim of developing and testing the effectiveness of a technique of fat harvesting and processing that would allow not only the procedure itself to be optimized and standardized, but would also offer the possibility of adding HCCs, in order to promote the establishment and development of adipocytes and ASCs, so improving the long-term outcome of the procedure.

Materials and methods

This preliminary experiment was performed on 13 patients after we had developed an automated closed-circuit fat washing system for lipofilling based on our expertise. It consists of two parts: laboratory evaluations and surgical procedure.

For laboratory evaluations, the adipose tissue of 3 female subjects, obtained by simple liposuction with a manual aspiration technique, processed by atraumatic cell washing in a closed system with added HCCs, was used.

In particular, a series of 3 samples was collected and examined for each patient:

- 20 mL of fat, without added HCCs;
- 20 mL of fat with 1 mL of HCCs (5%) added;
- 20 mL of fat with 2 mL of HCCs (10%) added.

The following laboratory tests were carried out on each sample:

- analysis of content in liquid and oily parts;
- phenotyping and cell growth tests on the stromal vascular fraction;
- analysis of metabolic viability of the adipocytes;
- microbiological tests.

The first *in vitro* evaluation was to analyze the liquid and oily content of the samples after centrifugation. In detail, 10 mL of sample from each preparation method was centrifuged at 400 g for 5 minutes at room temperature. After centrifugation, the different components, separated by density, were measured quantitatively.

The second and third laboratory tests were carried out on the ASCs. Specifically, phenotyping and cell growth tests were performed on the stromal vascular fraction (SVF). To phenotype the SVF, the samples processed with the washing system were exposed to the action of collagenases (type II, 0.1% final concentration) for 1 hour at 37°. The cell pellet was then washed with 5% PBS-FCS, before being passed through a 70 micron diameter pore filter and the cells were decorated with CD34 and CD90 antibodies and their respective isotypes (30 minutes incubation at room temperature, in darkness). These two markers were considered since CD34 is a marker of hematopoietic stem cells and ASCs, and CD90 is a marker of mesenchymal stem cells. For growth tests, on the other hand, isolated SVF was cultured on MesenCult medium for selection of mesenchymal stem cells and the degree of cell proliferation was evaluated after 8 days.

The fourth test consisted of analyzing the metabolic vitality of adipocytes. A free glycerol determination kit (Sigma Aldrich) was used to measure the release of free glycerol from the adipocytes to the adipose tissue of the washed samples, with and without the addition of HA. Glycerol release was determined after stimulation of adipocytes with isoproterenol by spectrophotometric reading at 570 nm.

The sterility of the samples was also tested using the following culture media: Columbia agar + 5% sheep blood, suitable for the growth of bacteria and yeasts; chromID CPS agar, suitable for the growth of gram positive and gram negative; chromID *Staphylococcus aureus* agar, suitable for the selective growth of staphylococci; Gélose chromID Candida, suitable for the growth of yeasts, in particular *Candida albicans*; broth culture BC0102M, for the inoculation of residual materials into a liquid culture.

The liquid material was sown in a sterile environment on the above-mentioned agar plates. The remaining material was inoculated into the broth culture as a liquid culture. In an independent series of tests, the proliferative capacities of the plates were verified by inoculating 0.05 McFarland of *Escherichia coli* (strain SL21Eco), *Staphylococcus aureus* (strainSL13Sau) and *Candida albicans* (strain SL-24Cal), all wild type from the laboratory collection. The plates were sealed and incubated for 15 days at 30 °C in a humidified thermostatic incubator.

In order to verify the tolerance and effectiveness of the association tested *in vitro*, 9 female and 1 male subjects were evaluated. After collecting autologous adipose tissue from areas such as the abdomen, hips, crural region and trochanteric region, depending on the availability in specific areas of accumulated fat in the treated patient, the lipoaspirate was washed and left to decant before being injected into the patient in the upper, middle and lower third according to the needs of the individual. Subsequently, the HCCs were injected. The follow-up was 6 months.

Results

After analyzing the percentage of adipose tissue contained in the samples, it was found that the sample treated with the closed-circuit washing system was the most concentrated in terms of percentage of adipose tissue (60%) compared to the control (55%). The sample purified with the same system and with the addition of 5% HCCs, although less concentrated (58%) than the sample without additions, was still more concentrated than the control (55%). The sample with 10% HCCs added presented adipocyte concentrations comparable to the control.

As regards the percentage of aqueous content in the samples, it was found that the sample treated with the washing system without additions of HCCs presented less total waste liquids (40%) than the control (45%), while those treated with FATStem with added HCCs presented higher percentages (43% for the 5% sample, 46% for the 10%), as was expected as a result of dilution with HCCs.

With regard to the percentage of free lipids contained in the samples, it was clear that all those treated with the closed circuit washing system, regardless of whether or not they had received added HCCs, presented lower levels of free lipid particles (equal to 5% for the sample without additions and that with 5% HCCs, and 10% for that with 10% HCCs) compared to the control (15%).

The phenotypic analysis of the SVF offered quite variable data in terms of percentage of positive CD34 and CD90 cells. In detail, the percentages varied according to the samples in a range between a minimum of 39.79% and a maximum of 74.32%. The highest percentages were recorded in the samples with added HCCs.

However, a difference between the samples with additions became clear: those with the addition of 5% HCCs showed a higher percentage of stem cells than those with 10% HCCs.

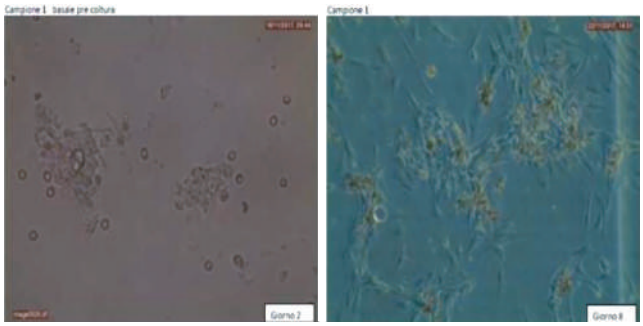

Figure 1.—SVF cell growth of adipose tissue not mixed with HCCs.

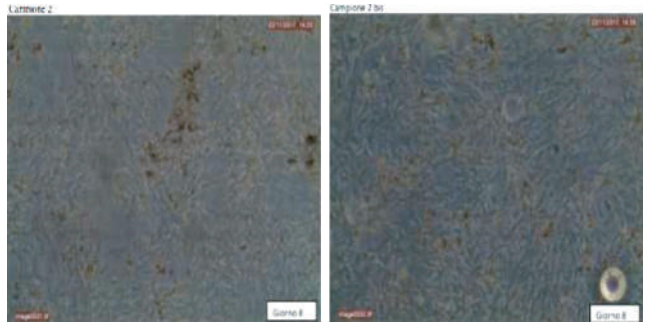

Figure 2.—SVF cell growth of adipose tissue mixed with 5% HCCs.

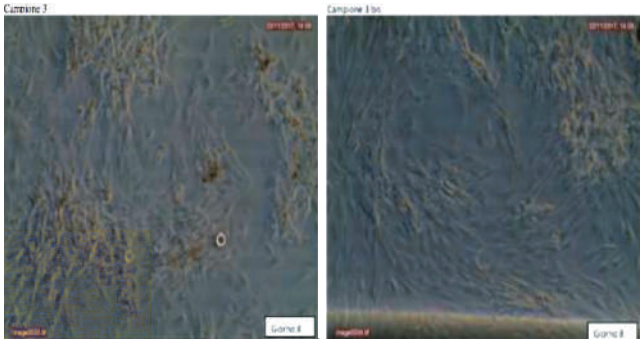

Figure 3.—SVF cell growth of adipose tissue mixed with 10% HCCs.

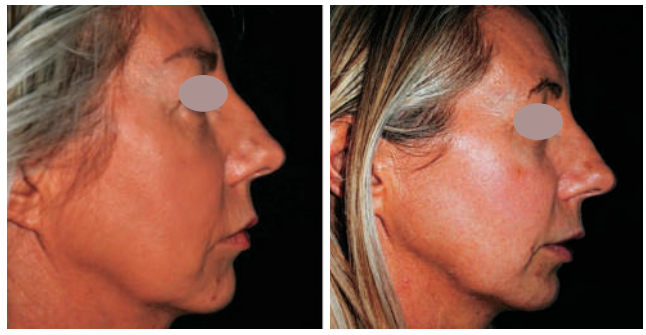

Figure 4.—Aesthetic result (before, left panel; after 6 months, right panel).

SVF cell growth tests have shown that the addition of HCCs promotes stem cell proliferation. In fact, after 8 days of culture, the stem cells of samples containing HCCs underwent a differentiation process in a much more evident way than the samples to which HCCs had not been added (Figure 1, 2, 3).

Analysis of the metabolic viability of adipocytes showed that cell viability was increased in adipose tissue samples with added HCCs, both at 5% and 10%.

Microbiological analysis at the end of the incubation period showed that the plates inoculated with materials derived from the closed circuit washing system disposables were all negative.

As far as the surgical part is concerned, even considering the small number of patients treated exclusively for general testing purposes for the completeness of the ongoing study, we can report our preliminary, subjective observations on the adequacy and potential of the association. At 6-month follow-up, in all cases subjected to this treatment protocol, an overall rejuvenating effect was obtained in addition to a satisfactory aesthetic result in terms of filling the treated areas of the face (Figure 4).

In the absence of signs of graft resorption, the skin in the treated areas appeared intact, distensible and elastic. Moreover, the good skin tone, which indicates good engraftment and revascularization, together with good trophism and skin tone, confirmed the success of the procedure clinically. No complications were recorded either during the surgical procedure or in the postoperative period.

Discussion

The fat processing system we chose for *in vitro* testing takes into account and responds to many of the problems to emerge from our bibliographic study.

The use of a closed system that reduces the risk of microbiological contamination during the procedural process and the use of an atraumatic cell purification system combine well with our need to use an automated system that makes the process less operator-dependent.

In fact, the parallel study on the components of the extracellular matrix and the development of new forms of tissue stimulation has allowed us to hypothesize a role of HA in this field too. In particular, HCCs could be a resource or even a fundamental factor in improving the performance of adipose grafting.

In vitro studies have shown a direct action of HCCs on adipose tissue as opposed to HA gels with different degrees of cross-linking that have not expressed any beneficial effect on adipose tissue. This result leads us to reflect on the regenerative efficacy of crosslinked fillers; the fact that free HA could positively influence not only fibroblasts and keratinocytes but adipocytes too could be

a plausible hypothesis, while the surprise was the superior activity of adipocyte preservation and stimulation on ASCs on the part of the latter.

There has been much discussion in the past about preserving fat by adding HA. The work presented in this preliminary experiment makes it possible to undertake an experimental path based on objective scientific findings, in fact the action demonstrated by *in vitro* HCCs can be transferred to the fat to be grafted as an optimization of modern lipofilling techniques. In fact, we can hypothesize that this new molecular structure of HA could be the ideal complement for a fatty graft able to perform mechanical and biological actions at the same time.

Shear stress with its cellular damage reduces the vitality of the adipocyte and its possible engraftment, while the rheological characteristics of the analyzed HCCs could reduce this physical damage through a fluidifying action in the implantation phase. A carrier such as this HA, which is moderately resistant to hyaluronidase, could protect the processed adipose mesenchymal tissue and facilitate its uniform distribution in the grafted tissues, promoting their engraftment. From a biological point of view, the properties and actions of this new molecular structure can be valuable as an ally both in the engraftment phase and in the subsequent phases of ASCs proliferation and maturation.

With this preliminary experiment we wanted to try to develop a simple and easily reproducible technique that would allow us to verify what had been previously hypothesized. We therefore validated a system of fat processing that would allow us to perform tests in order to optimize the implant product. The biological and physical tests were an indispensable aid for demonstrating viscoelastic suitability as well as the preservation of a suitable quantity of fat cells and ASCs.

The next step should be to clinically verify the effectiveness of the graft. However, we wanted to anticipate some observations by treating some areas of the face in selected patients with lipofilling and then with HCCs. The small number of patients treated has not allowed us to draw scientific conclusions but has allowed us to report, in advance, impressions that give us hope for a future standardized clinical phase.

Conclusions

Based on the results obtained, the efficacy of HCCs on the proliferation of ASCs and on the preservation of the vitality of adipocytes is confirmed. These premises can be considered optimal for a clinical trial on a larger number of patients, in order to confirm the value of the association of HCCs and autologous adipose tissue as a possible solution to the problem of graft resorption, which, in many cases, makes the results of lipofilling unsatisfactory in the long term.

References

1. Funt D, Pavicic T. Dermal fillers in aesthetics: an overview of adverse events and treatment approaches. Clin Cosmet Investig Dermatol 2013;6:295–316.

2. Kulichova D, Borovaya A, Ruzicka T, Thomas P, Gauglitz GG. Understanding the safety and tolerability of facial filling therapeutics. Expert Opin Drug Saf 2014;13:1215–26.

3. Stern R, Asari AA, Sugahara KN. Hyaluronan fragments: an information-rich system. Eur J Cell Biol 2006;85:699–715.

4. Kaya G, Rodriguez I, Jorcano JL, Vassalli P, Stamenkovic I. Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. Genes Dev 1997;11:996–1007.

5. Kogan G, Solte´s L, Stern R, Schiller J, Mendichi R. Hyaluronic acid: its function and degradation in in vivo systems. Bioactive natural products 2006;35.

6. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7:211–28.

7. Zielins ER, Brett EA, Longaker MT, Wan DC. Autologous Fat Grafting: The Science Behind the Surgery. Aesthet Surg J 2016;36:488–96.

8. Strong AL, Cederna PS, Rubin JP, Coleman SR, Levi B. The current state of fat grafting: a review of harvesting, processing, and injection techniques. Plast Reconstr Surg 2015;136:897–912.

9. Stellavato A, La Noce M, Corsuto L, Pirozzi AV, De Rosa M, Papaccio G, *et al.* Hybrid Complexes of High and Low Molecular Weight Hyaluronans Highly Enhance HASCs Differentiation: Implication for Facial Bioremodelling. Cell Physiol Biochem 2017;44:1078–92.

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

Manuscript accepted: April 17, 2019. - Manuscript received: February 5, 2019