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ORIGINAL RESEARCH ARTICLE

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Progenitor-cell-enriched micrografts as a novel option for the management of androgenetic alopecia

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Abstract

Regenerative medicine is a multidisciplinary field that combines engineering and life science principles to promote regeneration, potentially restoring the physiological condition in diseased tissues. Specifically, the developments of complex grafts enhance the intrinsic regenerative capacity of the host by altering its environment. Autologous micrografts obtained through Rigenera® micrografting technology are able to promote derma and bone regeneration. Androgenetic alopecia (AGA) leads to a progressive thinning of scalp hair affecting 60–70% of the adult population worldwide. Pharmacological treatment offers moderate results and hair transplantation represents the only permanent treatment option. The aim of this study was to demonstrate the role of dermis micrografting in the treatment of AGA by clinical and histological evaluations after 4, 6, and 12 months. Hair growth and density were improved at all indicated times. Those outcomes were also confirmed by the TrichoScan® analysis, reporting an increase of total hair count and density with an increase and reduction of anagen and telogen phases, respectively. Scalp dermoscopic analysis showed an improvement of hair density and histological analysis indicated a clear amelioration of the scalp, development of hair follicles, and a beginning of cuticle formation. Collectively, those results suggest a possible use of the micrografts as a novel therapeutic option in the management of AGA.

KEYWORDS

androgenetic alopecia, micrograft, regenerative medicine

1 | INTRODUCTION

Androgenetic alopecia (AGA) is characterized by the presence of several nonfunctional but still-alive hair follicles, referred to as "miniaturized follicles" and their amount is directly related with the severity of the disease (Yazdabadi, Magee, Harrison, & Sinclair, 2008). Clinically, AGA is a hereditary androgen-dependent condition, characterized by a progressive thinning of scalp hair, followed by a defined pattern that affects 60–70% of the adult population worldwide up to 40% of women and 50% of men by the age of 50 (Varothai & Bergfeld, 2014). The pharmacological treatment of AGA constitutes a multibillion-dollar industry frequently offering only moderate results. Such treatments also require the chronic application to achieve the continued benefit, with the risk of developing adverse side effects (Santos, Avci, & Hamblin, 2015) and strongly decreasing patient compliance. For this reason, hair transplantation represents the only current successful permanent treatment option, even if it requires a fairly invasive surgical procedure (Jain & De-Eknamkul, 2014; Levy & Emer, 2013). Actually, hair transplantation can be performed by two principal techniques, follicular unit transplant and follicular unit extraction, both are widely described in the literature (Jiménez-Acosta & Ponce-Rodríguez, 2017; Saxena

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& Savant, 2017). Another approach widely used is the injection of autologous platelet-rich plasma (PRP), which is able to reduce swelling and pain while increasing hair density (Ferneini, Beauvais, Castiglione, & Ferneini, 2017; Li et al., 2012).

The Rigenera® technology is an innovative clinical approach to obtain in one surgical time autologous micrografts able to promote derma and bone regeneration. This technology allows the mechanical disaggregation of small tissue samples previously harvested from the patient. Lastly, the device preforms also a selective filtration, collecting only the cells and tissue particles smaller than 80 μm. At the end of the procedure, which lasts only 2 min, the micrografts are collected from the reservoir located at the bottom of the Rigenera device. The regenerative role of micrografts was also reported in several in vitro studies, and more important, displayed high positivity to mesenchymal stem cells markers such as CD73, CD90, and CD105. Therefore, the obtained autologous micrografts were identified as progenitor-cell-enriched micrografts (Monti et al., 2017; Trovato et al., 2015). In the past, a preliminary study on three patients that underwent hair transplantation reported that the autologous micrografts obtained by Rigenera® were able to promote continuous hair growth even 2 months after the procedure with a shortening of the dormant phase of the follicle and faster healing of the microwounds. The authors postulate that the micrograft induced a strong and selective regenerative effect on the scalp of the patients (Zanzottera, Lavezzari, Trovato, Icardi, & Graziano, 2014). Based on these evidence, the aim of this study was to demonstrate the efficacy and safety of the progenitor-cell-enriched micrograft in the management of AGA. The clinical evaluations were performed after 4, 6, and 12 months from the micrografts application. Lastly, we have also performed histological and dermoscopic evaluation after 6 and 9 months from treatment highlighting the biological effect of the micrografts in a patient affected by AGA.

2 | SUBJECTS AND METHODS

2.1 | Subjects

AGA diagnoses were established based on a detailed medical history (i.e., screening for drugs linked to hair loss), clinical examination, and trichoscopic features (i.e., >20% variability in hair diameter between affected and unaffected areas). Subjects were clinically diagnosed with AGA upon presentation of an increase in miniaturized terminal hair and/or a reduced number of hair after physical examination and phototrichograms, along with negative hair pull tests. A total of 100 patients were treated according to the criteria defined before. Exclusion criteria were allergy to lidocaine, healing issues, chronic drug treatment, oncologic processes, and the execution of hair loss treatment between 3 months prior and 3 months after the date of applying treatment (except taking vitamin supplements and applying topical lotions or shampoos).

This study protocol is in accordance with the Declaration of Helsinki and the European regulations. All the patients provided written informed consent before participating in the study.

2.2 | Micrografts collection

The micrografts were prepared using the innovative medical device Rigeneracons (CE certified class I; Human Brain Wave, Turin, Italy). A 3 mm punch biopsy was used to extract 3 scalp tissues samples (Figure 1a) disaggregated by Rigeneracons adding 1.5 ml of sterile physiologic solution to the device (Figure 1b,c). The rotation of

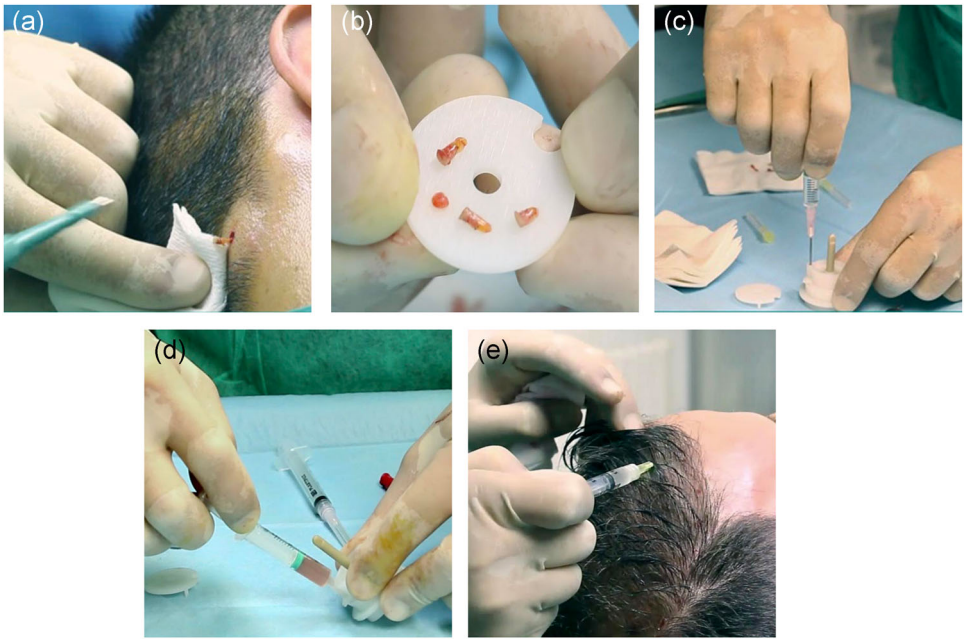

FIGURE 1 Procedure to collect autologous micrografts. (a), Punch biopsy to extract scalp tissue. (b, c) The strips of scalp tissue are placed into the Rigeneracons medical device adding 1.5 ml of sterile physiologic solution. (d) Collection of micrografts suspension by syringe without needle. (e) Infiltration of micrografts into the scalp of the patients

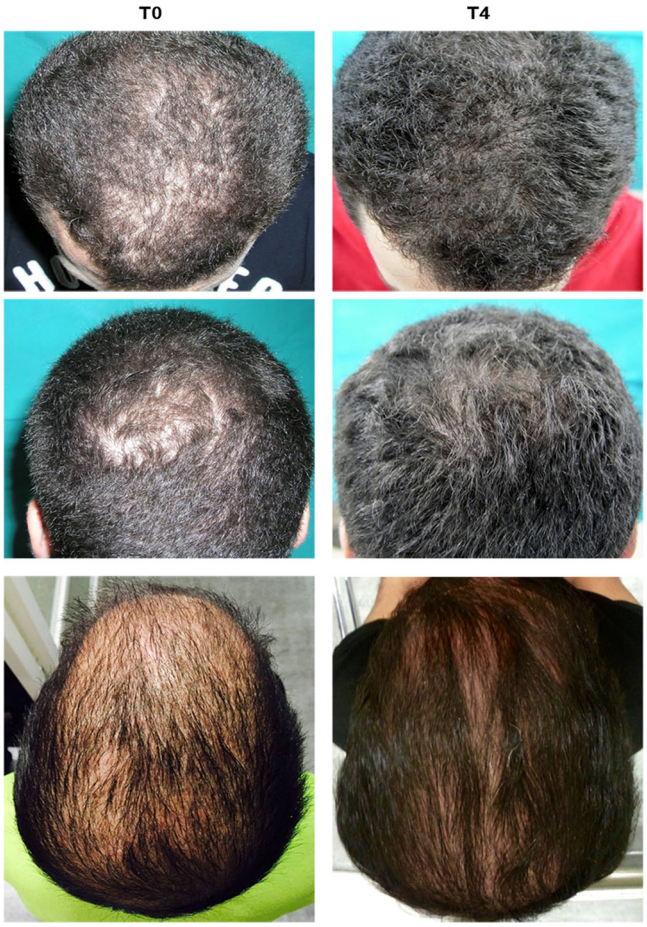

FIGURE 2 Three different representative cases of male patients before and after 4 months from micrografts application

Rigeneracons at 80 RPM by using the Rigenera machine for 2 min allows us to obtain the micrografts that were collected (Figure 1d) and directly infiltrated into the scalp of the patients as a mesotherapy (Figure 1e).

2.3 | TrichoScan® test

TrichoScan® (Teachscreen Software; Fotofinder, Bad Birnbach, Germany) is a computer-assisted dermoscopy with dedicated software to diagnose the hair loss and to measure its severity. Miniaturization is a pivotal requisite for establishing the AGA diagnosis. We randomly performed this test on 10 patients, as previously described (Riedel-Baima & Riedel, 2009). Briefly, an area of approximately 1 cm^2 on the parietal scalp was clipped with approximately 1 mm length. After 48 hr, a hair dye over that area was applied rinsing it 20 min later with a diluted alcoholic solution. Afterward, a microscopic image at ×20 was taken and analysis with TrichoScan® of the area was performed.

2.4 | Clinical evaluations

All the patients were evaluated at time 0 before the treatment, and after 4, 6, or 12 months from treatment and the effects of autologous micrografts on hair growth were assessed with the help of global photography, physician's and patient's global assessment scale.

2.5 | Histological evaluation of biopsies

Scalp biopsies were collected before and after 6 or 9 months from micrografts treatment, using a 4-mm punch probe from volunteer subjects. The biopsies were taken from the occipital region of the scalp, immediately fixed in 10% formalin and embedded in paraffin block to be cut and mounted onto microscope slides for analysis (Hashimoto et al., 2000). Biopsies were sectioned with ordinary microtome into 5-μm sections both vertically (parallel to the long axis of hair follicle) and transversely (perpendicular to the long axis of follicle and parallel to epidermis down toward subcutis). Histological sections were mounted on a glass slide sequentially and stained by Mallory's trichrome staining, as previously described, to visualize the collagen of connective tissue (highlighted by a blue coloration; Heidari et al., 2016). In addition, follicles were characterized as anagen, catagen, and telogen.

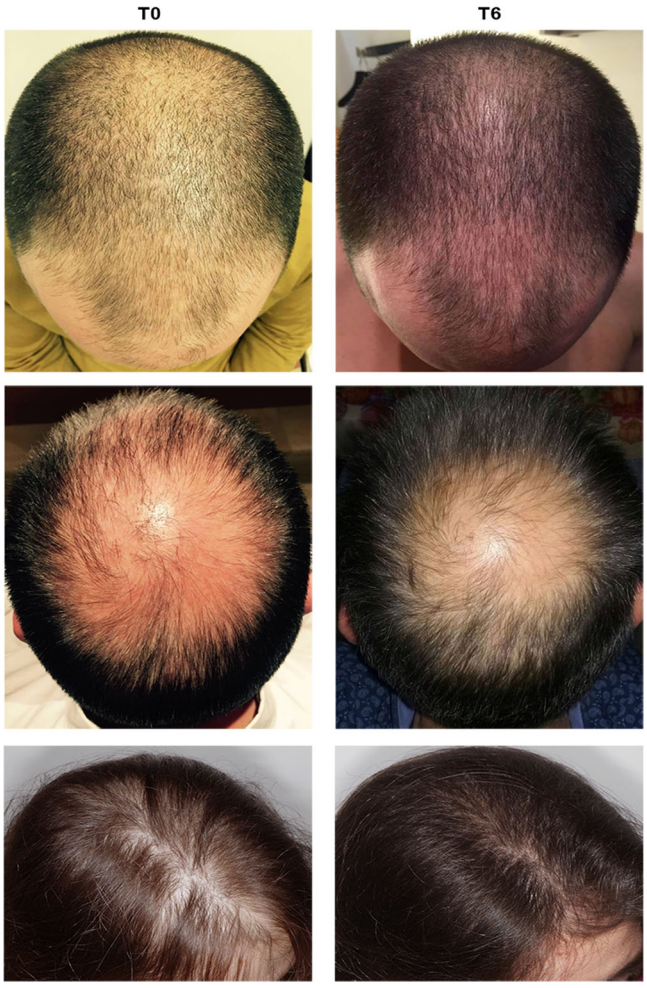

FIGURE 3 Three different representative cases of two male and one female patients before and after 6 months from micrografts application

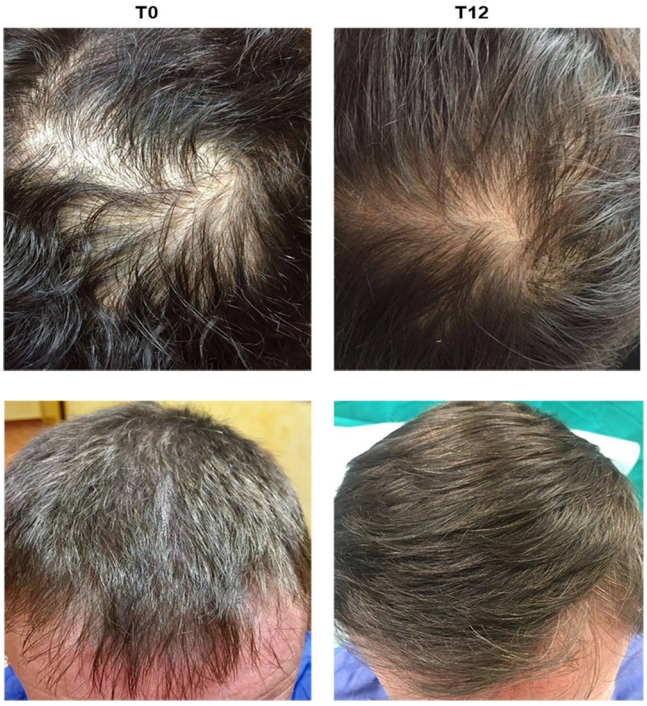

FIGURE 4 Two different representative cases of two male patients before and after 12 months from micrografts application

3 | RESULTS

The clinical evaluations of the use of the micrografts in the treatment of AGA were performed after 4, 6, and 12 months from micrografts application reporting significant improvement of hair growth and density at all indicated times (Figures 2–4). The visual results are confirmed by TrichoScan® analysis as reported in Table 1 (representative for a large number of patients) where it can be observed an increase of total hair count and hair density accomplished by an increase and reduction of anagen and telogen phases, respectively, after the micrografts application. The mean increase in total hair density was of 30% ± 3.0% already after 2 months of treatment compared with baseline values for the treated area. In addition, scalp dermoscopic analysis also showed an improvement of hair density after both 4 and 6 months from treatment (Figure 5).

Histological evaluations were performed by Mallory's trichrome staining on scalp biopsies at baseline and after 6 and 9 months from micrografts application. Figure 6 is representative of a large number of scalp biopsies. In the upper panels at baseline, the morphological appearance of the scalp is characterized by few hair follicles, lymphocytes infiltration, and prevalence of adipose tissue (Figure 6a,b). After 6 months of micrografts application, the number of hair follicles is higher with the beginning of cuticle formation and dermal papilla in proliferation (Figure 6c,d). In this case, it is possible to visualize a significant improvement in the anagen III phase with respect to baseline. In the lower panels, we reported the results after 9 months from the micrografts application. At baseline, we can observe an irregular derma, characterized by thin thickness, thin dermal papilla, indicating a lacking cellular proliferation. In addition, few hair follicles in the anagen III phase were observed (Figure 6e,f). After 9 months, we reported a well-organized derma, more regular, structured collagen fiber, and hair follicles in Anagen IV/Mesanagen phase. We also observed thick dermal papilla and signs of cellular proliferation (Figure 6g,h).

TABLE 1 Representative evaluation of hair growth by Trhicoscan® assay (A) before and (B) after 2 months of treatment

A	
Trichogram	
Area ($cm^{2)}$	0.72
Total hair count	11.0
Hair density ($1/cm^{2)}$	15.2
Anagen hairs (%)	72.7
Telogen hairs (%)	27.3
Hair length median (mm)	0.69
Density vellus hairs ($1/cm^{2)}$	2.1
Density terminal hairs ($1/cm^{2)}$	13.2
Count vellus	1.5
Count terminal	9.5
Ratio vellus hairs (%)	13.6
Ratio terminal hairs (%)	86.4
B	
Trichogram	
Area ($cm^{2)}$	0.72
Total hair count	35.0
Hair density ($1/cm^{2)}$	48.5
Anagen hairs (%)	78.3
Telogen hairs (%)	21.7
Hair length median (mm)	0.65
Density vellus hairs ($1/cm^{2)}$	10.4
Density terminal hairs ($1/cm^{2)}$	38.1
Count vellus	7.5
Count terminal	27.5
Ratio vellus hairs (%)	21.4
Ratio terminal hairs (%)	78.6

4 | DISCUSSION

The hair growth cycle is composed of three stages, anagen, catagen, and telogen. The AGA is a pathological condition characterized by an unbalance between the anagen and telogen phases with a reduction and increase of these phases, respectively. For this reason, a therapeutic approach able to stimulate hair follicles growth is commonly suggested. Many hair growths promoting therapies, such as minoxidil, PRP, and topical steroids were already identified (Chueh et al., 2013). Currently, minoxidil and finasteride are the only Food and Drug Administration (FDA) approved drugs; low-level laser light therapy is the only FDA-cleared device for the treatment of AGA and a

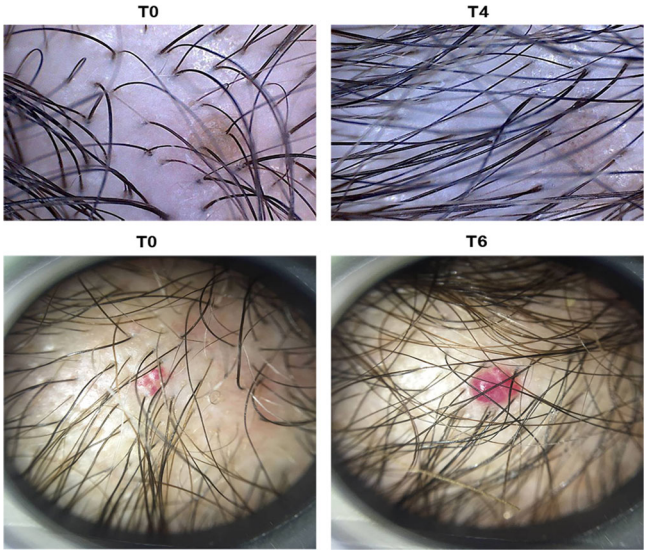

FIGURE 5 Scalp dermoscopic analysis performed by TrichoScan® before and after 4 and 6 months from micrografts application

recent published meta-analysis confirms the efficacy of these treatments with respect to placebo (Adil & Godwin, 2017). In addition to pharmacological treatment, there is a growing interest in alternative treatment strategy such as the use of growth factors or stem cells (Giordano, Romeo, & Lankinen, 2017; Park et al., 2010; Yoo et al., 2010). However, the use of stem cells is complex from the regulatory prospective and often not permitted. The use of the Rigenera devices allowed physicians and researchers to disaggregate the patient's own tissue, collecting progenitor-cell-enriched micrograft. Progenitors are a population of cells, resident in many tissues in the body, addressed to the regeneration, when possible, of the same tissue after injuries. Such cells express some of the stem cells' markers; however, differently from stem cells, progenitor cells are often unipotent and with limited capabilities of self-renewal (Seaberg & van der Kooy, 2003).

In this study, we have reported the effect of autologous micrografts on hair follicles restoration showing an improvement of hair growth and density after treatment, as demonstrated by photographs and TrichoScan® analysis. In accordance to these

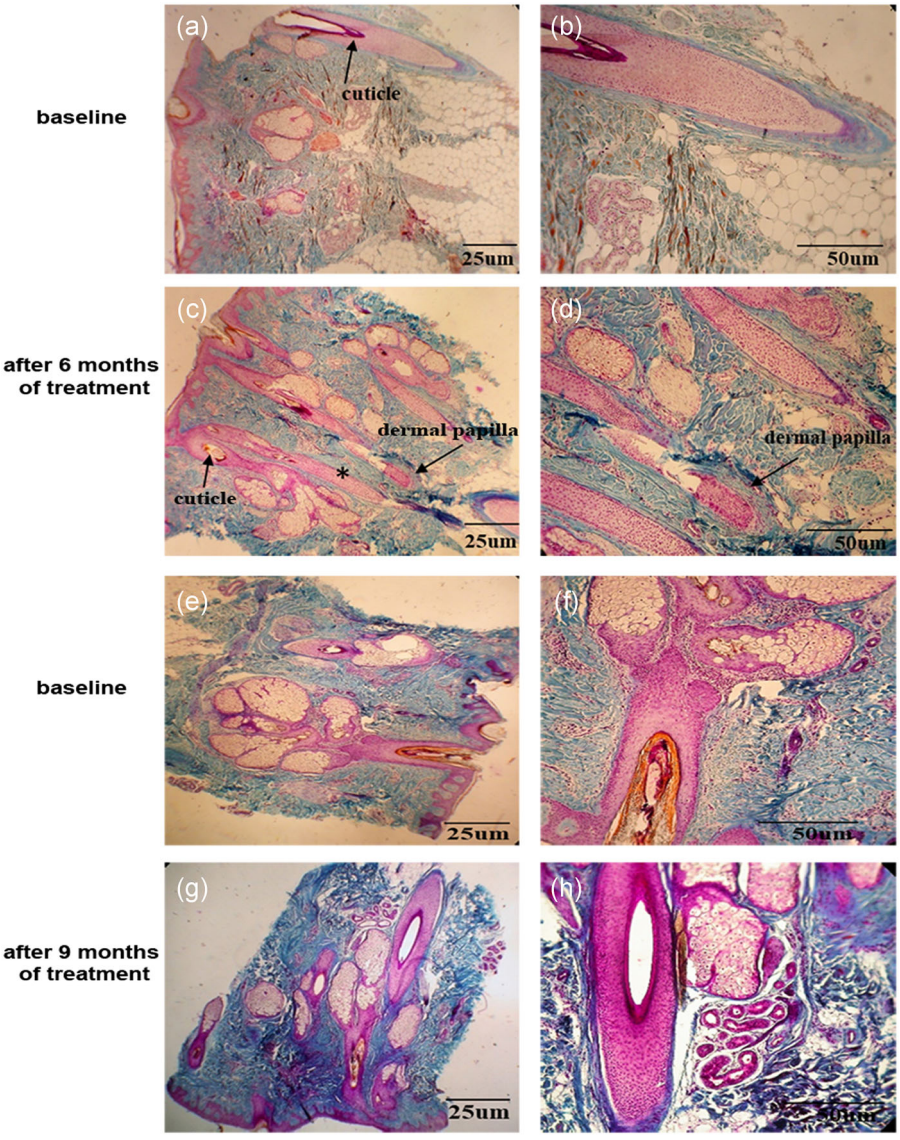

FIGURE 6 (a, b, e, and f) Histological evaluation of the occipital region of the scalp at baseline and after (c and d) 6 (g and h) and 9 months from micrografts application. The biopsies sections were stained by Mallory's trichrome. The symbol * indicates the presence of lymphocytes infiltration in the hair follicle. Magnification ×4, scale bar = 25 μm; magnification ×20, scale bar = 50 μm

data, a recent published study showed an increase of hair density after 6 months from treatment with human follicular cells isolated by the Rigenera® device without the need of culture conditions (Gentile, Scioli, Bielli, Orlandi, & Cervelli, 2017). In the same study, the authors also reported a good percentage of hair follicle CD44+cells, from the dermal papilla, and hair follicle epithelial CD200+cell, from the bulge suggesting their role in the hair follicle growth.

To confirm the effectiveness of autologous micrografts, other recent studies reported an increase of hair thickness together with a reduction of hair loss and a good patient's level of satisfaction after micrografts application. Furthermore, the treatment was well tolerated concerning pain without side effects (Álvarez, Valenzuela, & Tuffet, 2017; Álvarez, Valenzuela, & Tuffet, 2018).

In addition, the regenerative potential of the micrografts and the clinical efficacy have been already shown for bone regeneration (Brunelli et al., 2013; Rodriguez et al., 2017), in the treatment of ulcers (De Francesco et al., 2017; Miranda, Farina, & Farina, 2018; Trovato, Failla, Serantoni, & Palumbo, 2016), wound dehiscences (Baglioni, Trovato, Marcarelli, Frenello, & Bocchiotti, 2016; Marcarelli, Trovato, Novarese, Riccio, & Graziano, 2017), pathological scars (Svolacchia, De Francesco, Trovato, Graziano, & Ferraro, 2016), and lastly for cartilage and cardiac regeneration (Ceccarelli et al., 2017; Lampinen, Nummi, Nieminen, Harjula, & Kankuri, 2017; Gentile, Scioli, Bielli, Orlandi, & Cervelli, 2016).

Histological analysis performed in this study is suggestive for the role of micrografts in the development of new hair follicles (beginning of cuticle formation, increase of hair follicles, and decrease in adipose tissue). Based on the regenerative properties of micrografts, we can suppose two probable mechanisms for this effect. The first is the ability of micrografts to stimulate the quiescent hair follicular units reverting the miniaturization process typical of AGA and the second is the ability to induce the development of new hair follicles. Nevertheless, further and focused in vitro studies are needed to assess and confirm these suggestions. After the micrografts application, it was reported an improvement in hair restoration accomplished by a positive patient's subjective assessment. Taken together, these results are promising for major use of micrografts in the treatment of AGA, even if controlled and randomized clinical trials, with a larger sample, control, and placebo groups are necessary to confirm these effects.

CONFLICT OF INTERESTS

The authors Carlo Astarita and Antonio Graziano are members of Scientific Division of Human Brain Wave, the company owner of Rigenera TM technology. The other authors declare no competing interests.

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Orignial Article

Stem Cell INVESTIGATION

Stem cells from human hair follicles: first mechanical isolation for immediate autologous clinical use in androgenetic alopecia and hair loss

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Background: Hair follicles are known to contain a well-characterized niche for adult stem cells: the bulge, which contains epithelial and melanocytic stem cells. Stem cells in the hair bulge, a clearly demarcated structure within the lower permanent portion of hair follicles, can generate the interfollicular epidermis, hair follicle structures, and sebaceous glands. The bulge epithelial stem cells can also reconstitute in an artificial *in vivo* system to a new hair follicle.

Methods: In this study, we have developed a new method to isolate human adult stem cells by mechanical centrifugation of punch biopsy from human hair follicles without culture condition. Here, we used human follicle stem cells (HFSCs), to improve the hair density in 11 patients (38 to 61 years old) affected by AGA in stage 3–5 as determined by the Norwood-Hamilton classification scale.

Results: The primary outcomes were microscopic identification and counting of HFSCs. The secondary outcomes were clinical preliminary results and safety and feasibility in HFSCs-treated scalp. Each scalp tissue suspension contained about 3,728.5±664.5 cells. The percentage of hair follicle-derived mesenchymal stem cells CD44+ [from dermal papilla (DP)] was about 5%+0.7% whereas the percentage of hair follicle epithelial stem cells CD200+ (from the bulge) was about 2.6%+0.3%. In total, 23 weeks after the last treatment with HFSCs mean hair count and hair density increases over baseline values. In particular, a 29%±5% increase in hair density for the treated area and less than a 1% increase in hair density for the placebo area.

Conclusions: We have shown that the isolated cells are capable to improve the hair density in patients affected by androgenetic alopecia (AGA). These cells appear to be located in the bulge area of human.

Keywords: Human hair follicles stem cells; hair follicle stem cells (HFSCs); stem cells in hair loss; alopecia; androgenetic alopecia (AGA); hair loss

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Introduction

Eighty percent of Caucasian men experience some degree of androgenetic alopecia (AGA) before age 70 (1). Current legitimate treatments for AGA include finasteride, minoxidil, and hair transplantation (2). The role of platelet rich plasma has been demonstrated in recent reports (3,4).

In AGA, the follicle miniaturization is accompanied by a decrease of anagen, with an increase in the percentage

of resting (telogen) hair follicles containing microscopic hairs in bald scalp (5). In addition to these intrinsic changes to the hair follicle, infiltrating lymphocytes and mast cells have been identified around the miniaturizing follicle (6), especially in the area of the stem cell-rich bulge area (7). In balding scalp, the number of hair follicle stem cells (HFSCs) remains intact, whereas the number of more actively proliferating progenitor cells markedly decreases (8). This suggests that balding scalp either lacks an activator or has an inhibitor of hair follicle growth.

Here, we used HFSCs, obtained by mechanical centrifugation of scalp's punch biopsy, to improve the hair density in 11 patients (38 to 61 years old) affected by AGA.

The study protocol complied with the Declaration of Helsinki, the European regulations and all patients provided written informed consent before participating in the study.

Current regulations

In order to understand the sense of the current European regulations it is necessary to differentiate between "minimal manipulation" and advanced cell therapy performed by "extensive manipulation, which involves complex techniques of bioprocessing of therapeutic cells.

Reference is made to the Regulation n.1394/2007 of the European Parliament (EC) and of the Council 13 November 2007 on medicines for advanced therapies, where the definition of 'bioprocess engineering products' is given. Here it is specifically said that this definition excludes those products that contain, or are made exclusively of, cells and non-vital human or animal tissues and that do not have pharmacological, immunological or metabolic action. Included among the advanced therapy pharmaceutical products are those used for gene and somatic cell therapy [Directive 2001/83/(EC), European Community, Annex I]. Cells and tissues are to be considered products of bioprocess engineering if they undergo 'considerable manipulation'.

The same regulation defines the difference between extensive and minimum manipulation, and lists, which are considered relevant, or not.

Manipulations that are not considered "bioprocess engineering" are: cutting, grinding, shaping, sterilization, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, separation, concentration or purification, filtration, lyophilisation, freezing, cryopreservation and nitrification.

The extensive manipulation of cells and tissues is a process that may lead to cell activation and/or a stimulation of cell proliferation and these are also considered "extensively manipulated" cells that, although not specifically activated or stimulated to proliferate, are associated with biomaterials.

All cells that have undergone a manipulation of their genes are considered to be "extensively manipulated".

According to reflection paper on classification of advanced therapy medicinal products draft agreed, 20 June 2014 EMA/CAT/600280/2010 Rev 1, Committe for Advanced Therapies (CAT), Line 10 "*The same essential function for a cell population means that the cells when removed from their original environment in the human body are used to maintain the original function in the same anatomical or histological environment*", the authors resume that autologous use in one step surgery, minimal manipulation, monofunctional use "used for the same essential function in the recipient as in the donor", manipulation with devices in aseptic conditions, are conditions that do not require Good Manufacturing Practices (GMP) rules for processing, Good Clinical Practices (GCP) for the clinical application and Ethical Committee approval.

Methods

Patients

This study enrolled male patients who displayed AGA in stage 3–5 as determined by the Norwood-Hamilton classification scale. Additional exclusion factors were set based on systemic and local criteria. Specifically, systemic criteria for exclusion included evidence of sepsis, immunosuppression and cancer, as well as use of pharmacological therapeutics targeting AGA (i.e., finasteride, dutasteride, or antiandrogens) in the previous 12 months. Localized exclusion criteria included use of topical treatments for AGA (i.e., minoxidil, prostaglandin analogs, retinoids, or corticosteroids) in the previous 12 months and withdrawal of informed consent.

AGA diagnoses were established on the basis of a detailed medical history (i.e., screening for drugs linked to hair loss), clinical examination, and trichoscopic features (i.e., >20% variability in hair diameter between affected and unaffected areas). Patients were clinically diagnosed with AGA upon presentation of an increase in miniaturized terminal hair and/or a reduced number of hairs on physical examination and phototrichograms, along with negative hair pull tests. Laboratory tests were performed to exclude alternative causes of hair loss, such as poor nutrition, anemia, thyroid dysfunction, and syphilis. Urinalysis was used to detect levels of 17-idrocorticosteroid, 17-ketosteroid,

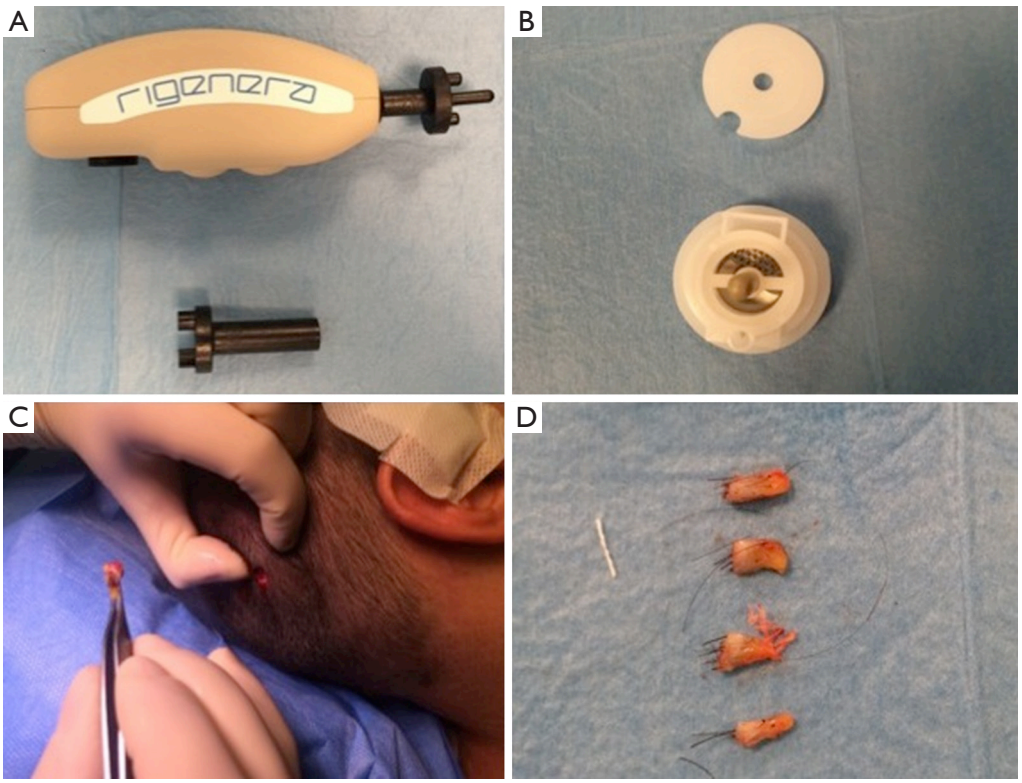

Figure 1 Rigenera procedure phase 1 (punch biopsy and cutting of scale tissues). (A) Rigenera Securdrill Device; (B) Rigeneracons kit; (C) the extraction of the scalp tissues during punch biopsy; (D) the authors cut the scalp tissues into the strips (2 mm × 2 mm).

dehydroepiandrosterone, free cortisol, pregnanetriol, and testosterone in all participants. Finally, circulating levels of cortisol, dihydrotestosterone, DHEA, D4-androstenedione, 17-hydroxyprogesterone, 3-α-diol glucuronide, prolactin, and gonadotropins were measured on all participants.

Human autologous hair follicle suspension procedure and preparation

Autologous suspension of HFSCs for immediate clinical use were prepared using an innovative medical device called Rigeneracons (CE certified class I, HBW srl; Turin, Italy) (*Figure 1A*,*B*). After the extraction of the scalp tissues during punch biopsy (*Figure 1C*), the authors cut the scalp tissues into the strips (2 mm × 2 mm) (*Figure 1D*) eliminating the excess adipose tissue. The strips were gently collected and disaggregated under sterile conditions (vertical laminar flow hood) by Rigeneracons (*Figure 2A*,*B*) in 1.2 mL of physiologic solution [NaCl 0,9% (mE/mL: Na^+ 0.154; Cl^- 0.154); mOsm/L 308, pH 4.5–7.0] (*Figure 2C*). After 60 seconds of centrifugation at 80 RPM per minute (*Figure 2D*), the cell suspension was collected from the system (*Figure 3A*,*B*) and mechanically infiltrated into the scalp of the patients affected by AGA (*Figure 3C*,*D*). In addition, the cell suspension obtained was cultured and subsequently characterized by cytospin and immunocytochemistry to identify the HFSCs.

The aim was to disaggregate a small piece of scalp tissue and opportunely select a cell population with a size of 50 µm.

Human autologous hair follicle suspension protocol and injection

For each patient, the scalp affected by hair loss was divided into four areas (frontal, parietal, vertex, and occipital); local anesthesia was not injected in the treated areas. Interfollicular HFSCs injections (0.2 mL·cm^2) were administered to select areas of the scalp at a depth of 5 mm using an Ultim gun (Anti-Aging Medical Systems, Montrodat, France) equipped with a 30-gauge (*Figure 3D*), 1 mL Luer lock syringe in two sessions spaced 60 days apart.

In patients with hair loss localized to the frontal

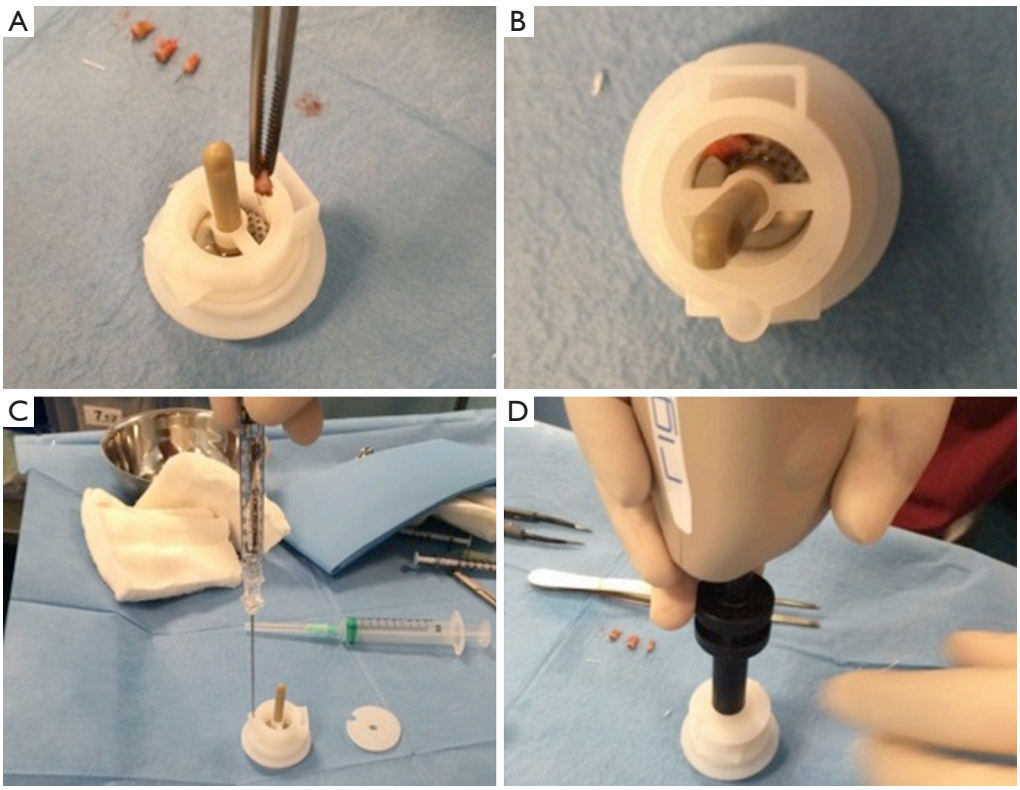

Figure 2 Rigenera procedure phase 2 (positioning of scalp tissue in Rigeneracons and centrifugation). (A) The strips collected into Rigeneracons; (B) detail of Rigeneracons containing one strip; (C) the addition of 1.2 mL of physiologic solution; (D) centrifugation at 80 RPM with Rigenera Securdrill device for 60 seconds.

and parietal regions, HFSCs injections were delivered exclusively to the frontal scalp while placebo injections (i.e., physiological saline) were injected in the parietal regions. Likewise, for patients with hair loss limited to the parietal and vertex regions, HFSCs was injected in the parietal region, and placebo was injected in the vertex region of the scalp. Equivalent numbers of autologous HFSCs and placebo injections were made.

Assessment of hair growth and clinical evaluation

All patients were evaluated in four stages: T0, beginning of study (*Figure 4A*); T1 in 3 weeks (*Figure 4B*); T2, in 9 weeks (*Figure 4C*); T3, in 16weeks and T4 in 23 weeks after the last treatment (*Figure 4D*). The hair growth evaluated after the last treatment was compared by photography with the baseline evaluation made before treatments and between the HFSCs treatment area and the control area, which received placebo injections. Photographs of the areas of a sample scalp treated with HFSCs are shown in *Figures 3C,5A*. The effects of HFSCs and placebo treatments on hair growth were assessed in all patients with the help of global photography (*Figure 5B*), physician's and patient's global assessment scale. In all patients, two translational areas of hair loss, one at the border of the treatment half and a second along the border of the placebo half, were demarcated with a semi-permanent tattoo.

Cytospin and immunocytochemistry procedures

Eleven samples of HFSCs suspension were analyzed in the Anatomic Pathology Institute of Tor Vergata University. Scalp tissue suspensions, fixed with 4% paraformaldehyde, were characterized for mesenchymal and epithelial stem cells markers, such as CD44 (9) and CD200 (10), respectively. After cell adhesion on a glass slide by cytospin, immunocytochemistry was performed with specific primary antibodies (CD44 sc-9960, 1:10; CD200 ab203887, 1:100).

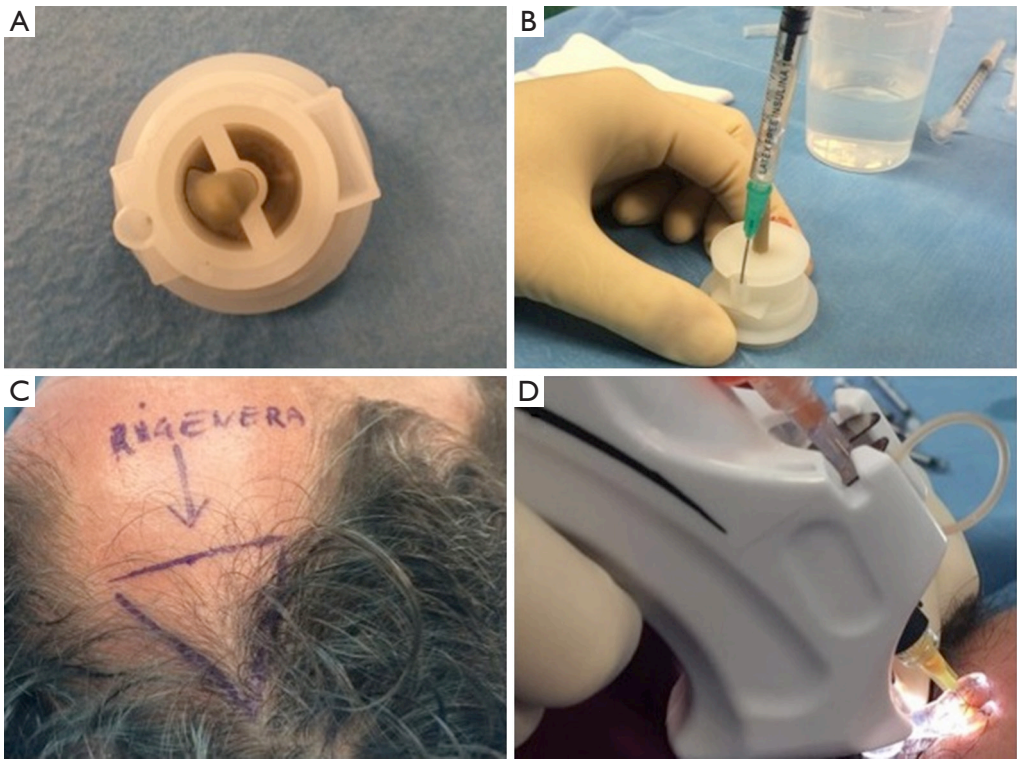

Figure 3 Rigenera procedure phase 3 (cell suspension contained in Rigeneracons and infiltration). (A) The cell suspension obtained by the system contained in Rigeneracons; (B) harvesting of cell suspension; (C) the selected area of the scalp treated; (D) mechanical and controlled infiltration performed by Ultim Gun.

Results

The primary outcomes were microscopic identification and counting of HFSCs. The secondary outcomes were clinical preliminary results and safety and feasibility in HFSCs-treated scalp.

Microscopic identification and counting of HFSCs

Each scalp tissue suspension contained about 3,728.5±664.5 cells. The percentage of hair follicle-derived mesenchymal stem cells CD44+ [from dermal papilla (DP)] was about 5%+0.7% (*Figure 6A*) whereas the percentage of hair follicle epithelial stem cells CD200+ (from the bulge) was about 2.6%+0.3% (*Figure 6B*). Positive cells were counted in the total area under a light microscope at 400× magnification (Eclipse E600, Nikon, Japan) and microphotographs captured by DXM1200F Digital camera (Nikon) using ACT-1 software (Nikon). The remaining cells were mainly represented by S100+ dermal fibroblasts (>85%) and epidermal cells (epithelial cells and melanocytes <10%) recognizable by their characteristic morphological aspects (data not shown).

Clinical results

In total, 23 weeks after the last treatment with HFSCs mean hair count and hair density increases (*Figure 4D*) over baseline values (*Figure 4A*). In particular, a 29%±5% increase in hair density for the treated area and less than a 1% increase in hair density for the placebo area. At the baseline, no statistical differences in hair count or hair density existed between the HFSCs treatment area and control area of the scalp.

In this preliminary report, we showed the clinical effect of the injection of scalp tissue suspension. However, we hypothesize that stem cells can improve the formation of new follicles, but this hypothesis must be demonstrated in a following study.

Discussion

The reconstitution of a fully organized and functional hair

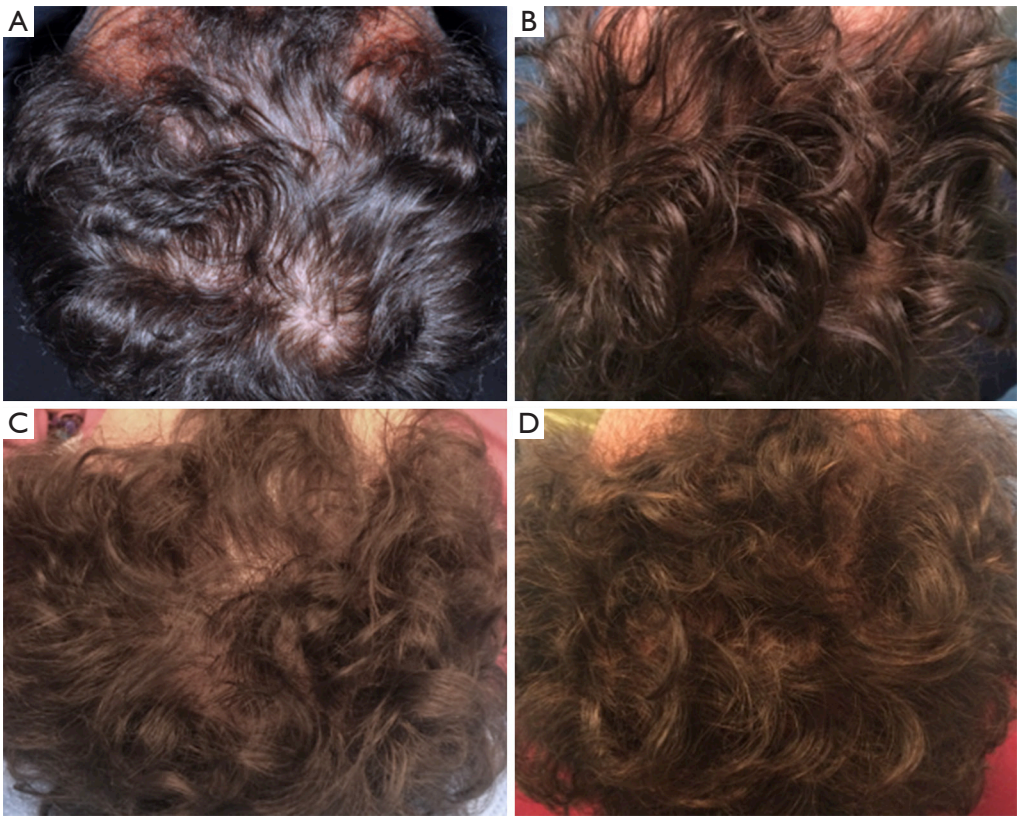

Figure 4 A smoker 45-year-old male patient affected by androgenetic alopecia classified AGA 3 according to Nordwood-Hamilton scale. (A) Preoperative situation at T0 of the scalp with hair loss localized to the parietal, temporal and frontal areas; (B) postoperative situation of the scalp at T1 after 3 weeks from the last treatment; (C) postoperative situation of the scalp at T2, after 9 weeks; (D) postoperative situation of the scalp at T4 after 23 weeks later the last treatment with increase of hair density.

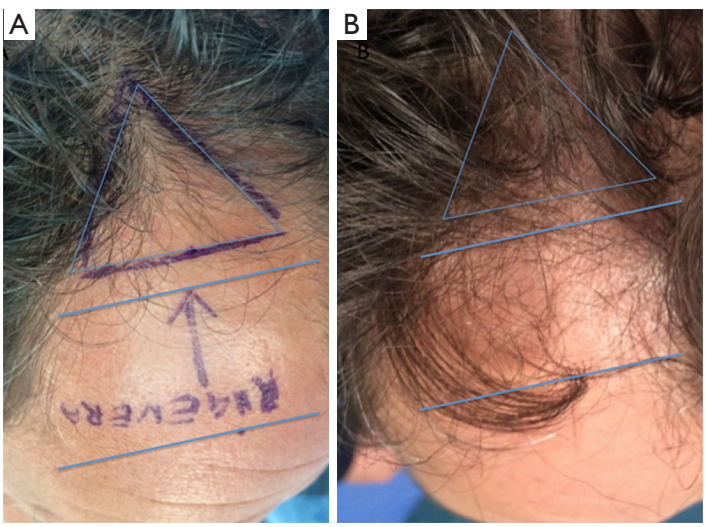

Figure 5 Detail of temporal right area of male patient affected by androgenetic alopecia classified AGA 3 according to Nordwood-Hamilton scale. (A) Preoperative situation at T0 of the scalp with hair loss localized to the right temporal and frontal area identified by blue lines; (B) postoperative situation of the scalp in the same area at T4 after 23 weeks later the last treatment with increase of hair density.

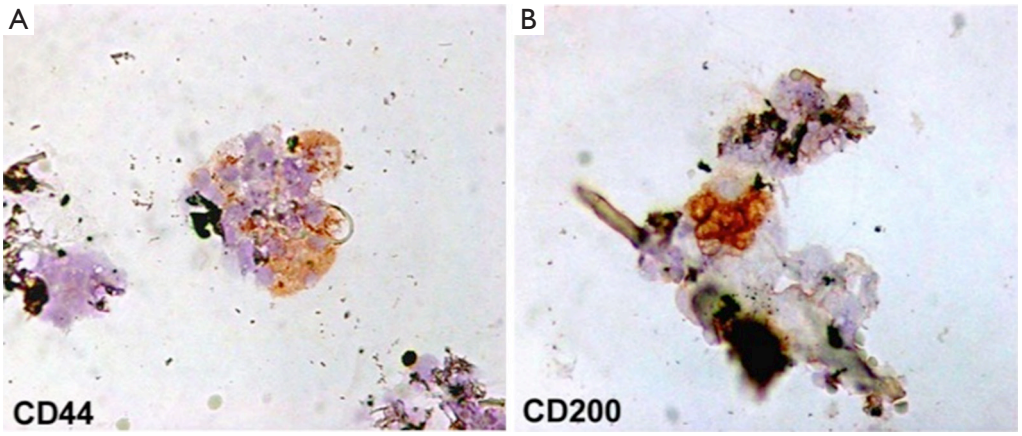

Figure 6 Immunophenotypic characterization of hair follicle stem cells in human scalp tissue suspension. Immmunocytochemistry for CD44 and CD200 stem cell markers. (A) hair follicle-derived mesenchymal stem cells; (B) hair follicle epithelial stem cells. Original magnification 400×.

follicle from dissociated cells propagated under defined tissue culture conditions is a challenge still pending in tissue engineering (11).

It is then of great interest to find different strategies aiming to regenerate or neogenerate the hair follicle under conditions proper of an adult individual. Based upon current knowledge on the epithelial and dermal cells and their interactions during the embryonic hair generation and adult hair cycling, many researchers have tried to obtain mature hair follicles using different strategies and approaches depending on the causes of hair loss (11).

In this preliminary study, the authors have developed a new method to isolate human adult stem cells by mechanical centrifugation of punch biopsy from human hair follicles without culture condition, and they reported for the first time, up to our knowledge, the counting of these cells and the preliminary results obtained by the human follicle stem cells injections in the scalp of patients affected by AGA, improving hair density.

In particular, the authors reported the percentage of hair follicle-derived mesenchymal stem cells CD44+, from DP, and the percentage of hair follicle epithelial stem cells CD200+, from the bulge.

The authors, now, feel the necessity discuss as follow, current advances in the different experimental strategies to regenerate or neogenerate hair follicles, with emphasis on those involving neogenesis of hair follicles in adult individuals using isolated cells and tissue engineering. Most of these experiments were performed using rodent cells, particularly from embryonic or newborn origin. However, no successful strategy to generate human hair follicles from adult cells has yet been reported. Perhaps the most important challenge is to provide three-dimensional culture conditions mimicking the structure of living tissue. Improving culture conditions that allow the expansion of specific cells while protecting their inductive properties, as well as methods for selecting populations of epithelial stem cells, should give us the necessary tools to overcome the difficulties that constrain human hair follicle neogenesis (11).

These cells appear to be located in the bulge area of human hair follicles. Hair follicles are known to contain a well-characterized niche for adult stem cells: the bulge, which contains epithelial and melanocytic stem cells (12). Stem cells in the hair bulge, a clearly demarcated structure within the lower permanent portion of hair follicles, can generate the interfollicular epidermis, hair follicle structures, and sebaceous glands (7,13). The bulge epithelial stem cells can also reconstitute in an artificial *in vivo* system to a new hair follicle (14,15).

The study published by Yu *et al.* (12) showed for the first time that human hair follicles also contain a stem cell population that can be differentiated into neuron, smooth muscle cell, and melanocyte lineages in induction medium. In addition, their data demonstrate that Oct4-positive cells are present in human skin, and most of them are located in the hair follicles *in vivo*. Oct4 belongs to the family of POU-domain transcription factors that are normally expressed in pluripotent cells of the developing embryo and mediate pluripotency (16).

It is possible that these Oct4-positive cells in the hair follicles are related to these pluripotent stem cells that can perceivably give rise to follicular melanoblasts, Merkel cells, and other cells. These stem cells might generate diverse cell types during tissue renewal or repair in response to environmental cues.

More research is warranted to further characterize these stem cells in the hair follicles. The hair bulge is a stem cell niche, which can be highlighted by K15 staining. Again, Yu *et al.* (12) demonstrated that most of the Oct4-positive cells in human skin are located in the areas highlighted by K15 staining *in vivo*, suggesting that these stem cells are located in the bulge area, an area that provides a unique differentiation-restricted environment for adult stem cells. In conclusion, their data indicate that human hair follicles contain multipotent stem cells other than epithelial and melanocytic stem cells, and these cells are located in the bulge area. These cells show promising plasticity in *ex vivo* and *in vitro* conditions, making them potential candidates for cell engineering and cell replacement therapies.

Human scalp tissues are easily accessible, and the fact that hair spheres can be generated from autologous adult tissue makes it an attractive source for individualized cell-based therapies.

Each mature hair follicle is a regenerating system, which physiologically undergoes cycles of growth (anagen), regression (catagen), and rest (telogen) numerous times in adult life (17). In catagen, HFSCs are maintained in the bulge. Then, the resting follicle re-enters anagen (regeneration) when proper molecular signals are provided. During late telogen to early anagen transition, signals from the DP stimulate the hair germ and quiescent bulge stem cells to become activated (18). Many paracrine factors are involved in this crosstalk at different hair cycle stages and some signaling pathways have been implicated (19-21). In anagen, stem cells in the bulge give rise to hair germs, then the transient amplifying cells in the matrix of the new follicle proliferate rapidly to form a new hair filament (22).

However, the cell dynamics in this process is less clear than in the physiological renewal and further studies are required to understand this process.

When the cellular niches are completely lost, it is necessary to generate a completely new hair follicle in a process called hair follicle neogenesis.

Based on the knowledge on the epithelial and dermal cells, and their interactions, during the embryonic hair generation and adult hair cycling, different experimental approaches have been designed to regenerate hair follicles or generate new ones by the neogenesis process. These hair regeneration and neogenesis attempts can be classified into four categories: (I) reversion of pathological intra- and/or extra-follicular environment, for instance AGA; (II) regeneration of complete hair follicles from the recombination of hair follicle parts; (III) neogenesis of hair follicles from isolated cells; and (IV) neogenesis of hair follicles by tissue engineering.

Regeneration of hair follicles was also observed in humans (23) when dermal sheath tissue was used, which was sufficient to regenerate also the DP structure. After implantation, the whisker DP was capable of inducing hair follicle regeneration retaining the information to determine hair fiber type and follicle size (24).

Grafting of dermal-inductive tissue was limited by the fact that it was not possible to generate more hair follicles than the obtained from the donor tissues. To overcome this limitation different approaches and experimental models using freshly or cultured isolated cells from both dermal and dermal/epidermal origin were tested. Most of them involved neonatal and embryonic murine cells.

In recent study published in 2015 by Balañá *et al.* (11) the authors prepared in a laboratory a dermal-epidermal skin substitute by seeding an acellular dermal matrix with cultured hair follicle epithelial stem cells and dermal papillar cells (DPCs), both obtained from adult human scalp. These constructs were grafted into a full-thickness wound generated on nude mice skin. In fourteen days, histological structures reminiscent of many different stages of embryonic hair follicle development were observed in the grafted area. These structures showed concentric cellular layers of human origin, and expressed k6hf, a keratin present in epithelial cells of the companion layer. Although the presence of fully mature hair follicles was not observed, these results showed that both epithelial and dermal cultured cells from adult human scalp in a dermal scaffold were able to produce *in vivo* structures that recapitulate embryonic hair development.

The analysis of all these studies could lead to the conclusion that hair follicle neogenesis using human epithelial and dermal cells is a very difficult task that could requires special culture conditions, somehow recreating the normal or embryonic skin environment, and the use of embryonic or neonatal cells.

Really, in more of 50 years, great progress was reported, starting from early 60s, to arrive now to april 2017, in

which, contrary to what appeared to emerge from previous studies, we have reported the last clinical advancement in the possibility to use human follicle stem cells obtained by mechanical centrifugation, respecting the European rules, without culture or use of enzymes, for AGA treatment.

Conclusions

Our preliminary data suggest that the injection of HFSCs preparations has a positive therapeutic effect on male androgenic alopecia without major side effects. Therefore, the authors recommend future study and clinical trials incorporate more data about the use of HFSCs.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The Ethical Committee approval is not required and written informed consent was obtained from all patients.

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Article

Platelet-Rich Plasma and Micrografts Enriched with Autologous Human Follicle Mesenchymal Stem Cells Improve Hair Re-Growth in Androgenetic Alopecia. Biomolecular Pathway Analysis and Clinical Evaluation

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Abstract: Platelet rich plasma (PRP) and Micrografts containing human follicle mesenchymal stem cells (HF-MSCs) were tried as a potential treatment for androgenetic alopecia (AGA). However, little to no work has yet to be seen wherein the bio-molecular pathway of HF-MSCs or PRP treatments were analyzed. The aims of this work are to report the clinical effectiveness of HF-MSCs and platelet-rich plasma evaluating and reviewing the most updated information related to the bio-molecular pathway. Twenty-one patients were treated with HF-MSCs injections and 57 patients were treated with A-PRP. The Wnt pathway and Platelet derived-growth factors effects were analyzed. 23 weeks after the last treatment with mean hair thickness increments (29 ± 5.0%) over baseline values for the targeted area. 12 weeks after the last injection with A-PRP mean hair count and hair density (31 ± 2%) increases significantly over baseline values. The increment of Wnt signaling in Dermal Papilla Cells evidently is one of the principal factors that enhances hair growth. Signaling from mesenchymal stem cells and platelet derived growth factors positively influences hair growth through cellular proliferation to prolong the anagen phase (FGF-7), inducing cell growth (ERK activation), stimulating hair follicle development (β-catenin), and suppressing apoptotic cues (Bcl-2 release and Akt activation).

Keywords: micrografts; HF-MSCs; human follicle mesenchymal stem cells; PRP; platelet rich plasma; hair loss; hair-regrowth; PRP hair; stem cells hair

1. Introduction

A clinical need exists for the development of biotechnologies to improve the hair re-growth in androgenetic alopecia (AGA).

AGA is a dynamic and chronic hair loss disorder, affecting 80% of white men and 40% of women before age 70, in which lymphocytes and mast cells have been seen around the miniaturizing follicle detailed in the stem cell-rich lump zone [1–4]. Miniaturization of the follicles is characterized by a diminishment of anagen phase, with an improvement in the amount of resting hair follicles, telogen, containing microscopic hairs in a hairless scalp [5–7]

Current treatments affirmed for AGA include drugs as Finasteride, topical lotions such as Minoxidil, and surgery as hair transplantation [2]. In hair loss scalp, hair follicle stem cell numbers stay unaltered, though the number of more actively proliferating progenitor cells particularly diminishes [8].

In this way, the aim of hair-tissue engineering (HTE) must be the development of new autologous-technologies to involve hair re-growth by in vitro and ex vivo culture or by in vivo regeneration and bio-stimulation. Autologous stem cells have been of great interest for application in hair-regrowth. Some early efforts in the field focused on isolating primary cells from a biopsy of the tissue of interest and growing the cells ex vivo for subsequent introduction back into the patient.

In the last year (2017), the authors reported the results of a medical device called Rigeneracons® (CE certified Class I, Human Brain Wave, Turin, Italy) to provide autologous micro-grafts enriched of human follicle mesenchymal stem cells (HF-MSCs) immediately available to be used in patients affected by AGA. The micrografts were obtained by the disaggregation of a 2 mm punch biopsy with the selection of a cell population with a diameter of 50 microns. High cell viability was reported. However, a major limitation encountered in this area has been the difficulty in expanding cells to sufficient numbers for human use, the necessity to perform this expansion in Good Manufacturing Practices (GMP) laboratories, and the viability of the expanded cells [9].

For this reason, the clinical use of HF-MSCs to improve hair re-growth has not been adequately considered.

In addition, the use of autologous platelets derived growth factors can represent valid support in hair-tissue regeneration (HTR) for their capacity to promote cell proliferation, differentiation and neo-angiogenesis, favoring the wound healing process [10–12]. In fact, platelet-rich plasma (PRP) contains at least six major growth factors, including basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and insulin-like growth factor-1 (IGF-1) released after platelet activation [11]. Each one of these major growth factors is involved in a specific bio-molecular activity.

The authors intended to clear up the clinical and bio-molecular impacts of HF-MSCs and PRP scalp infusion in people affected by AGA describing the bio-molecular pathway suggested in hair-regrowth. Information reviewed and reported exhibit the clinical efficacy and histological safety of the PRP and HF-MSCs treatments.

In this work, in particular, the recent advances of hair-tissue engineering using HF-MSCs, PRP, and new biotechnologies, were discussed reporting the most innovative results achieved, and the possible drawbacks or risks associated with the clinical translation of these technologies.

The examination convention agreed to the Declaration of Helsinki, the European and Italian rules. All patients gave written informed consent before partaking in the investigation.

2. Experimental Section

2.1. Rules and EMA/CAT Recommendations

This retrospective observational case-series study was conducted following the principles outlined in the Declaration of Helsinki and internationally consented ethics in clinical research [13]. A quality

assessment was carried out based on the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) checklist [14].

The protocol was conducted in strict adherence to the European Rules represented by Regulation n.1394/2007 of the European Parliament (EC) and by the Reflection Paper on characterization of cutting edge treatment medicinal products draft concurred, 20 June 2014 EMA/CAT/600280/2010 Rev 1, Committee for Advanced treatments (CAT), in which the autologous use in one step surgery, minimal manipulation, omofunctional utilization "utilized for an indistinguishable fundamental capacity in the beneficiary as in the donor", manipulation with gadgets in aseptic conditions, are conditions that do not require Good Manufacturing Practices (GMP) rules for processing, Good Clinical Practices (GCP) for the clinical application and the Ethical Committee endorsement. PRP preparation must be performed respecting in Italy "Decree of the Blood, 2 November 2015", dispositions related to quality and safety parameters of blood and emocomponents. All patients gave written informed consent.

The study protocol, object of a research contract (D.R. 1467/2017) and an university master's degree called "Regenerative surgery and medicine in wound care management", was approved with Rectoral Degree (D.R. n. 1794/2018) of 19 September 2018 and the Ethics on Research Committee of the School of Medicine, "Tor Vergata" University, Rome, Italy, with registration number #0031036/2018.

All patients received detailed oral and written information about the study, including the risks, benefits and alternative therapies, and signed an informed consent form before any study procedures.

2.2. Bio-Molecular Pathway of Stem Cells and Growth Factors that Improve Hair Re-Growth

- HGF and HGF activator (secreted by DPC): Improve proliferation of follicular epithelial cells;
- EGF: Improves migration and growth of follicle ORS cells by activation of Wnt/β-catenin signaling;
- b-FGF: Improves the development of hairs' follicles;
- IL-6: Involved in WIHN through STAT3 activation;
- VEGF: Improves peri-follicular angiogenesis;
- TGF-β: Stimulates the signaling pathways that regulate hair cycle;
- IGF-1: Improves survival, migration, and proliferation of hair follicle cells;
- IGFBP-1 to -6: Regulates IGF-1 effects and its interaction with extracellular matrix proteins at the hair follicle level;
- BMP: Maintains DPC phenotype (crucial for stimulation of hair follicle stem cell);
- BMPR1a: Maintains the proper identity of DPCs (essential for specific DPC function);
- M-CSF: Involved in wound-induced hair re-growth;
- M-CSFR: Involved in wound-induced hair re-growth;
- PDGF and PDGFR-β/-α64: Up-regulate the genes involved in hair follicle differentiation. Induction and regulation of anagen. PDGF and its receptors are essential for follicular development;
- Wnt3a: Involved in hair follicle development through β-catenin signaling;
- PGE2: Stimulates anagen in hair follicles;
- PGF2α: and analogs improve the transition from telogen to anagen;
- BIO (GSK-3 inhibitor);
- PGE2 or inhibition of PGD2 or PGD2 receptor D2/ GPR4477: Improve follicle regeneration;
- Iron and L-lysine (Under investigation)

2.3. Clinical Study Overview and Patients

Two works (HF-MSCs and A-PRP) were conducted and reviewed by the authors. This investigation enlisted 78 patients, 56 males who showed AGA in stage II–V as controlled by the Norwood–Hamilton classification scale and 22 females with AGA in stage I–II as dictated by the Ludwig classification scale. Twenty-one patients were treated with HF-MSCs injections and 57 patients were treated with A-PRP. Fundamental and local prohibition criteria were considered. Fundamental expulsion

criteria included immunosuppression and cancer, sepsis, and also the utilization of pharmacological therapeutics targeting on AGA (finasteride, similar drugs, and/or antiandrogens) in the earlier year. Localized expulsion criteria included the utilization of topical medicines for AGA in the earlier year. Low-level led therapy (LLLT) was proposed by the authors 15 days after each treatment to stimulate hair regrowth during the HF-MSCs and PRP treatment and every 3 weeks after the treatment until 6months post-treatment (Figure 1). The histological outcomes for patients treated with HF-MSCs and PRP were published by the authors. The Wnt pathway and platelet-derived-growth factors effects were analyzed and reviewed. AGA diagnoses were established on parameters reported in the previous works [3,4,15–17].

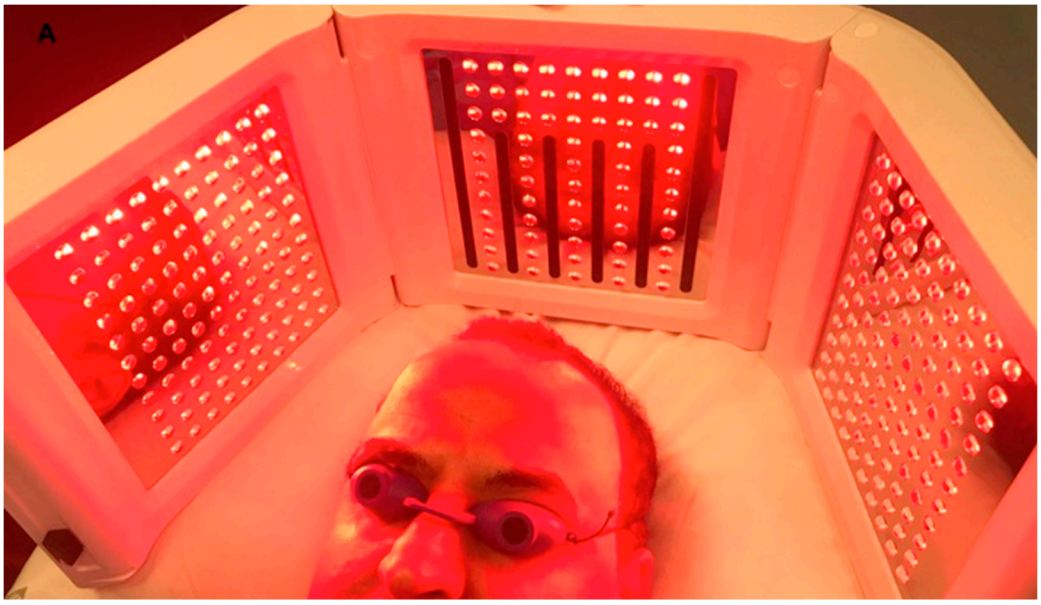

Figure 1. A non-smoker 58-year-old male patient classified androgenetic alopecia (AGA) 3V according to Norwood–Hamilton Scale. During Low level led therapy treatment performed by Geno-Led.

2.3.1. Preparation of Autologous Micro-Grafts Suspension

Autologous micro-grafts of HF-MSCs for human application were prepared using Rigeneracons (Medical Device with CE certified Class I, product by HBW Srl; Turin, Italy) (Figure 2A) in different steps. First step: harvesting of the scalp with punch biopsy (Figure 2B) and cutting the scalp tissues into the strips (2 × 2 mm) (Figure 2C,D); second step: collecting and disaggregation of the strips under sterile conditions (vertical laminar flow hood) performed by Rigeneracons (Figure 3B,C) in 1.2 mL of saline (NaCl 0.9%) (Figure 3A) through centrifugation at 80 rpm (Figure 3D) with the aim to select cells with a size of 50 µm; third step: collecting the micrograft's suspension from the system and mechanically infiltration, using 1 mL syringes into the selected area of the patient's scalp affected by AGA. Samples of micrograft's solutions obtained were previous cultured and subsequently characterized by cytospin and immunocytochemistry to identify the Human Follicle Stem Cells (HFSCs) (data published). The authors considered HFSCs the cellular population containing HF-MSCs and human hair follicle epithelial stem cells (HF-ESCs).

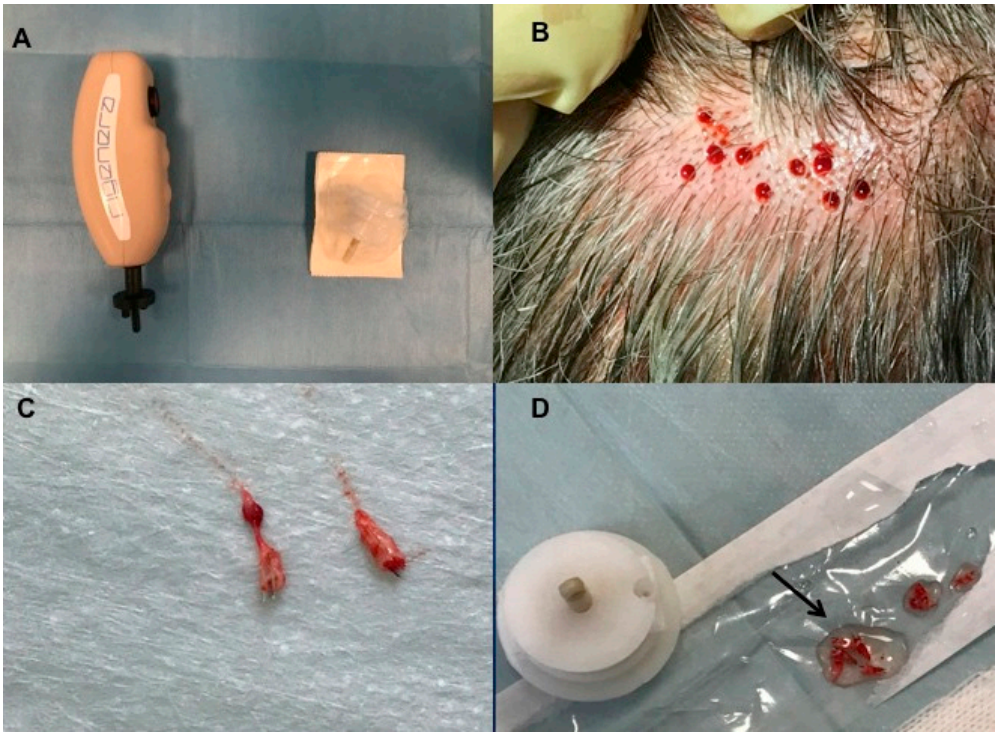

Figure 2. Micro-graft procedure phase 1. (**A**) On the left, Rigenera Securdrill device and on the right Rigeneracons kit; (**B**) The holes in the scalp after punch biopsy; (**C**) Selected scalp tissues into the strips (2 × 2 mm); (**D**) The authors controlled the presence of bulb in the selected tissue and conserved the strips into saline solution.

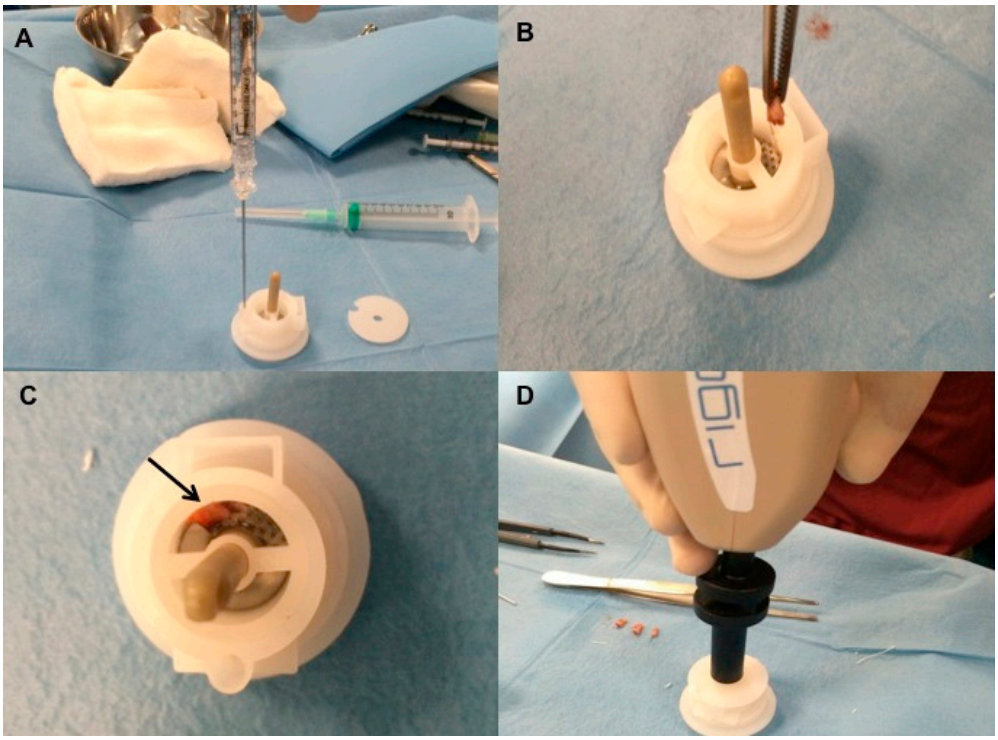

Figure 3. Rigenera procedure phase 2 (positioning of scalp tissue in Rigeneracons and fragmentation by centrifugation). (**A**) The addition of 1.2 mL of physiologic solution into Rigeneracons kit; (**B**) the strips collected into Rigeneracons; (**C**) Deatil of Rigeneracons containing one strip indicated by arrow; (**D**) centrifugation at 80 rpm with Rigenera Securdrill device for 60 s.

2.3.2. A-PRP Preparation and Delivery

Autologous blood (17.7 mL) was harvested using the i-Stem Kit PRP Preparation System (Figure 4A,B) (i-Stem, Biostems, Co., LTD., Seoul, South Korea 138-843, Medical Device, CE and Food and Drug Administration (FDA)) under the approval of the transfusional service. Sodium citrate (ACD) as an anticoagulant, was added (2.2 mL).

After the first spin (centrifugation at 3000 rpm for 6 min) (Figure 4C), the authors removed the Platelet-Poor Plasma PPP portion (1 mL) and RBC (Red blood cells) (2 mL) and re-centrifuged for the second time (3000 rpm for 3 min) and at the end of the procedure, 15 mL of A-PRP was obtained (Figure 4D–F). Microscopic platelet counts were performed on the A-PRP collected from all participants.

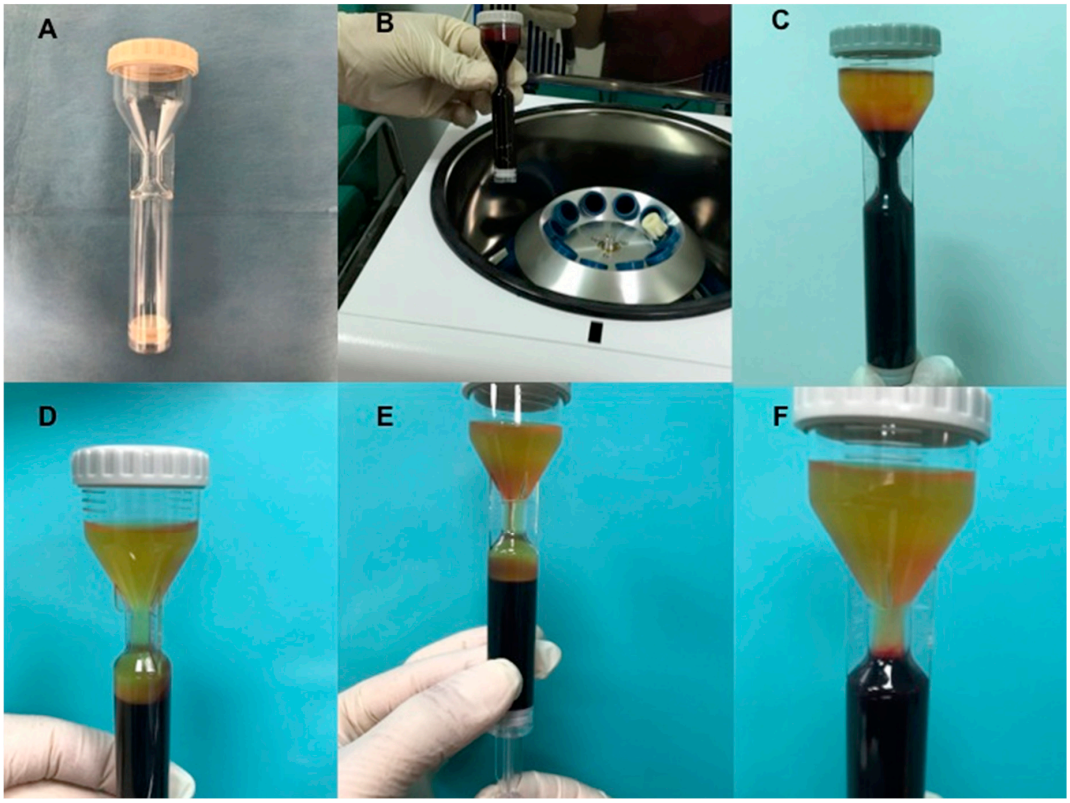

Figure 4. i-Stem platelet rich plasma (PRP) procedure. (**A**) Kit i-Stem; (**B**) The blood collected in i-Stem Kit underwent at centrifugation; (**C**) The PRP and PPP suspension obtained by the system after the first centrifugation; (**D**) The PRP and PPP after the second centrifugation.; (**E**) selection of PRP; (**F**) PRP concentration in the middle side of kit.

2.3.3. Mechanical and Controlled Injection (MCI) of Autologous Suspension and Study Design

In the patients treated with PRP or with HF-MSCs, the scalp was separated into six regions (as previous published). The interfollicular infusions, (0.2 mL × cm^2) were realized to targeted regions at a depth of 5 mm, with MCI procedure [17] utilizing an Ultim gun (Anti-Aging Medical Systems, Montrodat, France) outfitted with a 1 mL Luer lock syringe with needle 30-gauge, in two sessions spaced two months apart (relatively to HF-MSCs) and in three session spaced 30 days average using 10 mL Luer-look syringe (relatively to PRP).

Suspension infusions were conveyed to the frontal area while placebo infusions (i.e., saline NaCl 0.9%) were infused in the parietal areas in patients with hair loss confined to the frontal-parietal areas. In like manner, for hair loss restricted to the parietal-vertex regions, the suspension was infused in the parietal area, and saline was infused in the vertex area. Equivalent quantities of autologous micrograft's suspension and placebo infusions were made.

2.3.4. Evaluation of Hair Re-Growth

Assessment of hair growth was assessed in five phases: T0, before the first infusion (Figures 5A and 6A); T1 in 3 weeks; T2, in 9 weeks; T3, in 12 weeks (Figure 5B); T4, in 16 weeks, T5 in 23 weeks (Figure 6B), after the last treatment. The hair re-growth assessed after the last treatment was contrasted by picture and the baseline assessment made before medications and between the micrografts and/or PRP treatment region and the manipulation region, which got placebo infusions. The second infusion was performed following two months in the case of HF-MSCs injection. On the other hand, in the case of PRP treatment, the last infusion was the third and each injection was performed each 30 days average.

Photos of the area treated with a suspension of PRP are shown in Figure 5A,B. Photos of the area treated with a suspension of micro-grafts are shown in Figure 6A,B. The impacts of micrografts' and PRP's suspension and placebo medications on hair re-growth were evaluated in all patients with the assistance of worldwide picture, doctor's and patient's worldwide evaluation scale.

In all patients, two translational regions of hair loss, one at the fringe of the treatment half and a moment along the outskirt of the placebo half, were demarcated with a semi-permanent tattoo.

2.3.5. Statistical Analysis

Values as the mean in addition to baseline mistake were examined by means of Student's *t*-test, and contrasts considered statistically noteworthy were $p < 0.05$.

3. Results

3.1. PRP Clinical Results

As beforehand detailed [4,17], 12 weeks later the treatment with A-PRP mean hair count increases significantly over baseline values. In particular, the values obtained, reflect a 31 ± 2% increase in hair density for the treatment group and less than a 1% increase in hair density for the placebo group. Moreover, both the hair count and hair density parameters represent statistically significant improvements in hair growth for the A-PRP treated scalp (Figure 5B) over the placebo-treated control group.

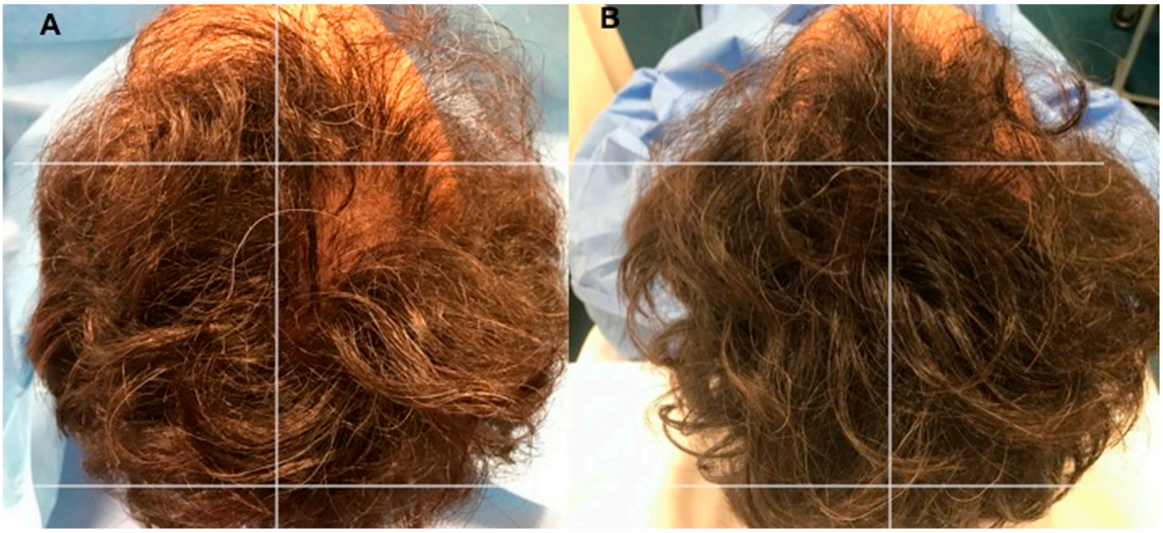

Figure 5. A non-smoker 34-year-old male patient classified AGA 2 according to Norwood–Hamilton Scale. (**A**) Timing T0 (before the treatment) with hair loss localized to the vertex, parietal, temporal, and frontal areas; (**B**) Timing T3 after 12 weeks later the last third treatment with increase of hair density in the right parietal area treated with three autologous platelet-rich plasma not activated (A-PRP) injections versus left parietal area treated with placebo.

As reported in our previous work [3,4] both patient populations treated with A-PRP (autologous-PRP not activated) and AA-PRP (autologous activated-PRP) respectively, showed an improvement in the number of follicular bulge cells and follicles, epidermal thickening, improved vascularization, and a higher number of $Ki67^+$ basal keratinocytes in PRP-treated scalp tissue compared with placebo.

Indeed, histological examination of A-PRP and AA-PRP treated scalp from our previous work [3,4] provides such clinical evidence.

Hair re-growth at the clinical level showed a similarly positive response to treatment with A-PRP, with patients manifesting significant improvements in hair count and total hair density in the targeted area over the control area (treated with Placebo). Differences between 12 weeks follow-up counts and baseline count for these hair growth parameters were higher in the A-PRP treatment population in this study than in the AA-PRP treatment population in the previous trial performed by the authors [3]. In particular, 3 months hair density measurements for patients treated with A-PRP and AA-PRP were 65 ± 5 and 28 ± 4 hairs/cm^2, respectively (Tables 1 and 2). The results obtained constitute a 31 ± 2% increase in hair density when A-PRP treatment is performed versus 19 ± 3% increase in hair density when AA-PRP treatment is performed, with a statistically significant difference in hair growth ($p = 0.0029$) (Tables 1 and 2). The increase of hair growth parameters for A-PRP over AA-PRP may reflect the greater efficiency of in vivo thrombin to activate platelets and the body to distribute the contents of activated platelets compared with in vitro calcium activation and injection. Moreover, delivery of A-PRP may enable the production of thromboxane A2 (TXA2) by the platelets once they are activated in vivo, which would activate additional platelets and amplify platelet aggregation [18].

3.2. Micrografts Clinical Results

12 weeks later the treatment with micro-grafts, mean hair density was increased significantly over baseline values. In particular, the values obtained, reflect a 30 ± 5.0% increase in hair density for the treatment group and less than a 1% increase in hair density for the placebo. Hair Density measurement for area treated with micro-grafts 12 weeks after the last treatment was 39 ± 5 hairs/cm^2 (Table 1).

As beforehand detailed [15], 23 weeks later the last treatment with micrografts containing HFSCs mean hair density increments (29 ± 5.0%) over baseline values for the treated region and less than a 1% increment in hair density for the placebo region (Figure 6B). At the baseline, no statistical contrasts in hair tally or hair thickness existed between the micrografts treatment region and manipulation region of the scalp.

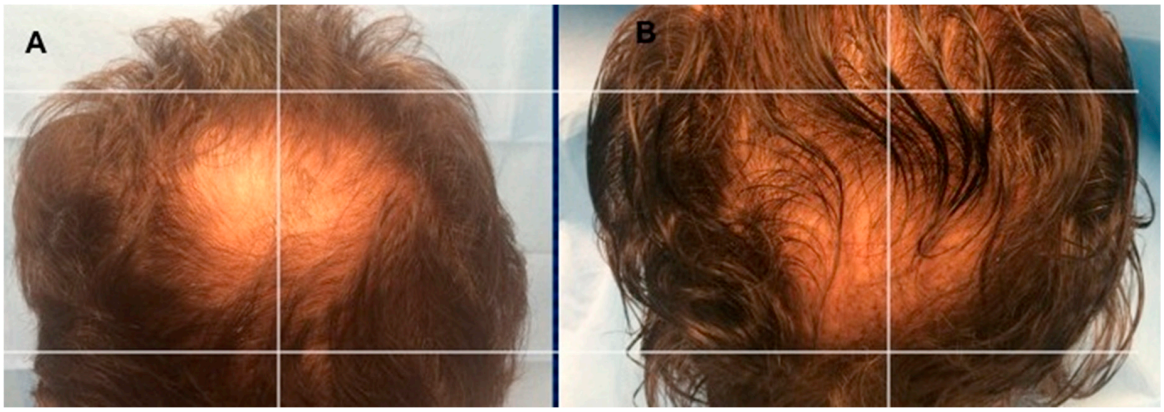

Figure 6. A non-smoker 54-year-old male patient classified AGA 4 according to Norwood–Hamilton Scale. (**A**) Timing T0 (before the treatment) with hair loss localized to the vertex; (**B**) Timing T5 after 23 weeks later the last second treatment with increase of hair density in the vertex area treated with two micrograft injections versus parietal area treated with placebo.

In the preparatory investigation [15], the authors have built up another strategy to disengage human grown-up stem cells by minimal manipulation based on centrifugation of strips of human hair follicles without expansion or culture. They reported the counting of these cells and the preparatory outcomes acquired by the human follicle stem cells infusions in the scalp of patients affected by AGA, enhancing hair thickness.

Specifically, in the past work, the authors detailed the amount (in term of percentage) of $CD44^+$ cells (hair follicle-derived mesenchymal stem cells), from dermal papilla, and the percentage of $CD200^+$ cells (hair follicle epithelial-stem cells), from the bulge, gotten via programmed centrifugation of 11 punch tests [15].

The authors revealed, the microscopic assessment performed by cytospin, immunocytochemistry, histological examination got by Hematoxylin-Eosin-stained and clinical assessment, feeling the need to talk about as follow, current advances in the distinctive methodologies to improve regeneration of hair follicles, with accentuation on those including neogenesis of hair follicles in grown-up people utilizing isolated cells and biotechnologies.

Table 1. Study design and clinical results obtained using Micrografts containing HFSCs and autologous platelet-rich plasma not activated (A-PRP).

Platelet-Rich Plasma	Micrografts
Injections performed in three session spaced 30 days	Injections performed in two sessions spaced 60 days
Mechanical and controlled injection	Mechanical and controlled injection
Addition of low-level led therapy (LLLT) 15 days after each treatment and every 3 weeks after the third treatment until 6 months post-treatment	Addition of low-level led therapy (LLLT) 15 days after each treatment and every 3 weeks after the second treatment until 6 months post-treatment
Hair density measurements for A-PRP (not activated-PRP) 12 weeks later the last treatment: 65 ± 5 hairs/cm^2	Hair density measurements for Micrografts 12 weeks later the last treatment: 39 ± 5 hairs/cm^2
Hair density improvement for A-PRP (not activated-PRP) 12 weeks, later the last treatment, compared with placebo area: 31 ± 2%	Hair density improvement for Micrografts, 12 weeks later the last treatment compared with placebo area: 30 ± 5.0%
Hair density improvement for A-PRP (not activated-PRP) 23 weeks, later the last treatment, compared with placebo area: 28 ± 2%	Hair density improvement for Micrografts, 23 weeks later the last treatment compared with placebo area: 29 ± 5.0%

Table 2. Clinical results obtained using A-PRP versus AA-PRP.

A-PRP (Not Activated)	AA-PRP (Activated)
Hair density measurements for A-PRP, 12 weeks later the last treatment: 65 ± 5 hairs/cm^2	Hair density measurements for AA-PRP, 12 weeks later the last treatment: 28 ± 4 hair cm^2
Hair density improvement for A-PRP 12 weeks, later the last treatment, compared with placebo area: 31 ± 2%	Hair density improvement for AA-PRP 12 weeks, later the last treatment, compared with placebo area: 19 ± 3%
Hair density improvement for A-PRP 23 weeks, later the last treatment, compared with placebo area: 28 ± 2%	Hair density improvement for A-PRP 23 weeks, later the last treatment, compared with placebo area: 15 ± 3%

4. Discussion

It is of extraordinary enthusiasm to discover distinctive methods planning to improve regeneration of the hair follicle under conditions appropriate of a grown-up person. In light of the current knowledge on the epithelial and dermal cells and their associations amid the embryonic hair generation and grown-up hair cycling, numerous researchers have endeavored to acquire mature hair follicles utilizing distinctive methods and methodologies relying upon the reasons for AGA [19–21].

Investigations were performed utilizing rat cells, especially from embryonic or infant origin. Notwithstanding, no successful methodology to generate human hair follicles from grown-up cells has yet been accounted for. Maybe the most critical challenge is to give three-dimensional culture conditions mirroring the structure of living tissue. Enhancing culture conditions that permit the extension of particular cells while securing their inductive properties, and additionally, the strategies for choosing populaces of epithelial stem cells should give us the fundamental tools to overcome the challenges that constrain human hair follicle neogenesis [21].

These cells give off an impression of being situated in the lump region of human hair follicles.

Hair follicles are known to contain a well-characterized niche for grown-up stem cells: the lump, which contains epithelial and melanocytic stem cells [22]. Stem cells in the hair lump, an obviously differentiated structure inside the lower permanent portion of hair follicles, can generate the interfollicular epidermis, hair follicle structures, and sebaceous glands [7,23]. The lump epithelial stem cells can likewise reconstitute in a simulated in vivo framework to a new hair follicle [24,25].

Yu et al. [22] demonstrated that follicles of human hairs contain a stem cell populace that can be separated into the smooth muscle cell, neuron and melanocyte heredities in induction medium. Their information demonstrates that $Oct4^+$ cells are available in the skin of a human, and the majority of them are situated in the hair follicles in vivo. Oct4 has a place with the family of POU-domain transcription factors that are regularly communicated in pluripotent cells of the developing embryo and mediate pluripotency [26]. Subsequently, follicles of human hair contain multipotent stem cells other than epithelial and melanocytic stem cells, and these cells are situated in the lump region. These cells indicate promising plasticity in ex vivo and in vitro conditions, making them potential candidates for cell engineering and cell substitution treatments.

Each mature hair follicle is a regenerating framework, which physiologically experiences cycles of growth (anagen), relapse (catagen), and rest (telogen) various times in grown-up's life [27]. In catagen, hair follicle stem cells are kept up in the lump. At that point, the resting follicle re-enters anagen (regeneration) when legitimate molecular signals are given. Amid late telogen to early anagen change, signals from the Dermal Papilla (DP) stimulate the hair germ and quiescent lump stem cells to wind up activated [28]. Numerous paracrine factors are engaged with this crosstalk at various hair cycle stages and some signaling pathways have been implicated [29–31]. In anagen, stem cells in the lump offer ascent to hair germs, at that point the transient increasing cells in the grid of the new follicle proliferate quickly to frame another hair filament [32]. As a matter of fact, the authors feel the need to better know which stage is critical to act.

Regeneration of hair follicles was likewise seen in people [33] when dermal sheath tissue was utilized, which was adequate to regenerate additionally the DP structure. After implantation, the whisker DP was equipped for promoting hair follicle regeneration holding the data to decide hair fiber type and follicle size [34].

In an examination reported by Balañá ME et al. [21] the authors prepared in a research facility a dermal-epidermal skin substitute by seeding an a-cellular dermal grid with cultured hair follicle epithelial stem cells and dermal papilla cells (DPCs), both gotten from the grown-up human scalp. These constructs were grafted into a full-thickness wound produced on bare mice skin. In fourteen days, histological structures reminiscent of a wide range of phases of embryonic hair follicle improvement were seen in the grafted region. These structures demonstrated concentric cellular layers of human origin and expressed k6hf, keratin present in epithelial cells of the companion layer. Despite the fact that the presence of completely mature hair follicles was not observed, these outcomes demonstrated that both epithelial and dermal cultured cells from the grown-up human scalp in a dermal scaffold could create in vivo structures that reiterate embryonic hair improvement.

In a recent report published in 2017 by Kalabusheva et al [35], the authors combined postnatal human DPCs and skin epidermal keratinocytes (KCs) in a hanging drop culture to build up a simulated hair follicle germ. The technique depends on DP cell hair-inciting properties and KC self-association. They assessed two protocols of total collecting. Blended HF germ-like structures showed the initiation of epithelial-mesenchymal collaboration, including WNT pathway enactment and expression of follicular markers. They examined the impact of conceivable DP cell niche components including dissolvable components and extracellular matrix (ECM) molecules during the time spent on the organoid assembling and growth. Their outcomes showed that soluble components had little effect on HF germ generation and $Ki67^+$ cell score inside the organoids despite the fact that BMP6 and VD3 kept up effectively the DP character in the monolayer culture. Aggrecan, biglycan, fibronectin, and hyaluronic acid (HA) significantly stimulated cell proliferation in DP cell monolayer culture with no

impact on DP cell character. A large portion of ECM compounds restricted the growth of cell totals while HA advanced the formation of bigger organoids.

Talavera-Adame et al [36], revealed in a recent study the bio-molecular pathway involved in a cellular treatment. Specifically, it has been additionally demonstrated that Wnt/β-catenin signaling is fundamental for the growth and upkeep of DPCs [37,38]. The increment of Wnt signaling in DPCs evidently is one of the principal factors that enhance hair re-growth [37].

In particular, in a study published by Pirastu N. et al [39], androgen receptor signaling, is implicated by seven genes at six loci. Three main groups were found: genes linked to Wnt signaling (RSPO2; LGR4; WNT10A; WNT3; DKK2; SOX13; TWIST2; TWIST1; IQGAP1; and PRKD1), genes involved in apoptosis (DFFA; BCL2; IRF4; TOP1; and MAPT) and a third more heterogeneous group including the androgen's receptor and TGF-β pathways (RUNX3; RUNX2; ALPL; PTHLH; RUNX1; AR; SRD5A2; PDGFA; PAX3; and FGF5). Although many different pathways have been implicated in the development of androgenetic alopecia, their results suggest that in addition to the androgen receptor pathway, for which they confirm a prominent function, the Wnt and apoptosis pathways play a fundamental role. Androgenetic Alopecia is characterized by a shorter growth (anagen), which has been associated with increased apoptosis of the hair follicle cells. This result suggests the anagen phase becomes shorter because of differences in the genes regulating apoptosis. The Wnt pathway has been implicated in the transition from the telogen (resting) to the anagen (growth), and also in the determination of the fate of the stem cells in the hair bulge, which are both dysregulated in balding tissue. Finally, baldness risk loci in the WNT ligand biogenesis and trafficking and Class B/2 (Secretin family receptors) pathways were also associated with height, despite none of the individual loci in these pathways being significant: this suggests a "pathway-wide" effect. Therefore, baldness shows pathway-specific genetic correlations, which provide a potential biological basis to observed epidemiological correlations. Pathway-specific genetic correlations hold promise in disentangling the shared biological pathways underpinning complex diseases [39].

Another fascinating field is the likelihood to utilize the fat graft and stromal vascular fraction cells (SVFs) in hair re-growth. SVFs is a heterogeneous group of non-cultured cells that can be dependably removed from fat by utilizing computerized frameworks, in light of centrifugation, filtration and purification of fat tissue or utilizing enzymatic absorption (not proposed by EMA-CAT recommendations). These cells work to a great extent by paracrine systems to help adipocyte viability. Festa et al [40] detailed that adipocyte ancestry cells bolster the stem cell niche and help drive the complex hair growth cycle. This follicular regenerative approach is fascinating and raises the likelihood that one can drive or reestablish the hair cycle in male and female hair loss by stimulating the niche with autologous fat improved with SVF.

Along these lines, Perez-Meza D et al. [41], detailed the safety, tolerability, and quantitative, in patients affected by hereditary alopecia treated with sub-cutaneous scalp infusion of advanced fat tissue. An increment of 31 hairs/cm^2 was recorded in patients experiencing treatment of fat blended with SVF; one subject who had fat alone reported a mean increment of 14 hairs/cm^2, proposing that while fat alone may represent an approach for early hairlessness, the addition of SVF may improve this reaction [41]. The discoveries propose that scalp stem cell-enriched fat grafting may represent a promising elective way to deal with treating hair loss in people. Fukuoka et al. [42] inspected the impacts of fat-derived stem cell-conditioned medium infusion in a group of 22 patients affected by AGA. Patients got treatment each 3 to 5 weeks for a total of 6 sessions. The mean increment in the hair count was 29 ± 4.1 in men and 15.6 ± 4.2 in women. No noteworthy distinction was seen amongst men and ladies.

The examination of the investigations reviewed could prompt the conclusion that hair follicle neogenesis utilizing human epithelial and dermal cells is an extremely cumbersome assignment that could require unique culture conditions, some way or another reproducing the ordinary or embryonic skin condition, and the utilization of embryonic or neonatal cells.

In the other hand, the number of papers published on PRP is considerable, but the results are often contradictory. The authors thinking that It is not correct to speak of PRP in general, but it is better to identify different types of PRP preparations depending on their cell content and fibrin architecture. On this way it is possible to identify:

1. Leukocyte-poor PRP (LP-PRP) or Pure Platelet-Rich Plasma (P-PRP). Suspension without leukocytes and with a low-density fibrin network after activation;
2. PRP and Leukocyte (L-PRP). Suspensions with leukocytes and a low densities fibrin network after activation (the largest number of commercial kit);
3. Leukocyte- poor platelet-rich fibrin (LP-PRF) or pure platelet-rich fibrin (P-PRF). Suspensions without leukocytes and a high-density fibrin network.
4. Leukocytes and platelet rich fibrin (L-PRF) or second generations PRP products are preparations with leukocytes and a high-density fibrin network.

As reported, there are too many protocols for preparation of PRP depending on the different time and RPM used, the number of platelets, the availability of growth factors and chemokines. There is also a wide biological (between patients) and temporal (day to day) variation [43]. So, it is difficult to assess which kit for PRP preparation is better and which is worse [44].

Different PRP products might be more or less appropriate to treat different types of tissues and pathologies. The clinical efficacy of PRP remains under debate, and a standardized protocol has not yet been established [45]. So, physicians should select proper PRP preparations after considering their bio-molecular characteristics and patient indications [46].

Recently, the use of low-level led therapy (LLLT) has been suggested as a treatment for AGA and to improve hair re-growth. LLLT was proposed by the authors 15 days after each treatment to stimulate hair regrowth during the HF-MSCs and PRP treatment and every 3 weeks after the treatment until 6 months post-treatment. Eleven studies were reviewed by Afifi et al. [47]. 9 studies assessing hair count/hair density found statistically significant improvements in both males and females following LLLT treatment. Additionally, hair thickness and tensile strength significantly improved in 2 studies. Patient satisfaction was reported in 5 works.

Autologous platelet-rich plasma (A-PRP), is also now associated with improved surgical outcomes and lower recurrence rates when incorporated in the treatment protocol for the gingival recession and keloid therapies, respectively [48,49].

In dermatological use, differences were found when PRP therapies were performed with the delivery of activated autologous PRP (AA-PRP) in place of non-activated A-PRP. When A-PRP is used with autologous thrombin to yield AA-PRP, it is possible to observe the healing of chronic wounds and shortened recovery times for deep burns [50,51]. Likewise, laser resurfacing of acne outcomes affords qualitatively better results with fewer side effects when performed in conjunction with either topical or intradermal application of calcium-activated PRP [52]. These results, probably, may be attributed to the release and concentration of alpha-granule proteins, including growth factors and cytokines, that stimulate cellular differentiation and proliferation, angiogenesis, and vascular modeling [53].

In the treatment of hair loss, topical use of AA-PRP to harvested follicles prior to implantation has already been shown to increase their survival rate by 15% [54].

Moreover, patients treated with calcium gluconate-activated PRP exhibit increased hair density three months post-surgery with terminal hair density (diameter > 40 μm) increasing by 19% during that time [16].

These findings were confirmed in a study following AGA patients treated with calcium-activated PRP over the course of one year [55]. Twelve weeks later the last injection of PRP, hair density peaked with a 19% increase over baseline measurements; at the one-year mark, hair density fell to 7% above baseline measurements but this value still constituted a significant increase in hair density compared to the baseline values [55].

The growth factors (GFs) obtained by the degranulation of the alpha-granules have been shown to stimulate hair re-growth. In detail, insulin-like growth factor-1 (IGF-1) stimulates proliferation of

cycling Ki67$^+$ basal keratinocytes [56,57], while transforming growth factor β1 (TGF-β1) protects the proliferative potential of basal keratinocytes by inhibiting cell growth and terminal differentiation [58,59]. Platelet-derived growth factor AA (PDGF-AA) increase the hair inductive activity of DPCs when applied in combination with fibroblast growth factor 2 (FGF-2) [60,61]. Vascular endothelial growth factor (VEGF) stimulates angiogenesis, and PDGF-BB is a potent chemo-attractant for wound macrophages and fibroblasts and stimulates these cells to release endogenous growth factors, including TGF-β1, that promote new collagen synthesis [62].

DPCs harvested from human scalp have shown increased proliferation, increased Bcl-2 and FGF-7 levels, activated ERK and Akt proteins, and up-regulation of β-catenin when cultured in an activated PRP-supplemented growth medium [63]. Since each of these factors positively influences hair re-growth through cellular proliferation to prolong the anagen phase (FGF-7) [28], inducing cell growth (ERK activation) [64], stimulating hair follicle development (β-catenin) [65], and suppressing apoptotic cues (Bcl-2 release and Akt activation) [66,67]. Human scalp affected by AGA injected with PRP should display marked increases in cellular activity. Indeed, histological examination of A-PRP and AA-PRP treated scalp from our previous works [3,17,18] provides such clinical evidence. In both patient populations, the authors observed improvement in the number of follicular bulge cells and follicles, epidermal thickening, improved vascularization, and a higher number of Ki67$^+$ basal keratinocytes in PRP-treated scalp tissue compared with placebo.

5. Conclusions

Our information obviously highlights the constructive effects of Micrografts-HF-MSCs and/or PRP infusions on AGA. Micrografts-HF-MSCs and PRP may fill in an effective and a safe procedure alternative to AGA; PRP and/or HF-MSCs represent a promising therapy for AGA. The increment of Wnt signaling in DPCs evidently is the important factor that enhances hair re-growth. DPCs have shown increased Bcl-2 and FGF-7 levels, activated ERK and Akt proteins, and upregulation of β-catenin when cultured in an activated PRP-supplemented growth medium.

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ORIGINAL RESEARCH

Short-Term Efficacy of Autologous Cellular Micrografts in Male and Female Androgenetic Alopecia: A Retrospective Cohort Study

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Purpose: Autologous cellular micrografts (ACM) is a novel treatment method in hair loss, and few data are available regarding its efficacy. The present study was carried out to assess the short-term clinical efficacy of a single application of ACM in the treatment of male and female androgenetic alopecia (AGA).

Materials and Methods: This was a single-center retrospective study involving 140 consecutive adults with confirmed AGA, who received a single session of ACM (Regenera Activa®). Efficacy was evaluated 1–6 months after treatment, by analyzing the change of trichometry parameters, which were assessed using TrichoScan digital image analysis.

Results: Depending on the scalp region, there was increase in mean hair density by 4.5–7.12 hair/cm^2, average hair thickness by 0.96–1.88 µm, % thick hair by 1.74–3.26%, and mean number of follicular units by 1.30–2.77, resulting in an increase of cumulative hair thickness by 0.48–0.56 unit. Additionally, the frontal region showed a significant decrease in % thin hair (−1.81%, p = 0.037) and yellow dots (−1.93 N/cm^2, p = 0.003). A favorable response was observed in 66.4% of the participants in the frontal region. Further, a gender-specific effect of treatment was observed.

Conclusion: ACM is a promising treatment in AGA with a short-term favorable response observed in up to approximately two-thirds of patients.

Keywords: androgenetic alopecia, pattern hair loss, autologous cellular micrografts, efficacy, regrowth

Introduction

Androgenetic alopecia (AGA) or pattern hair loss (PHL) is the most common cause of hair loss. It affects approximately 50% of men and women by the age of 50, and its prevalence increases with advancing age.[1,2] As the name implies, AGA has a clear genetic predisposition mediating an excessive response to androgens.[3]

The treatments for AGA are limited. Topical minoxidil is the only FDA-approved treatment in females, and topical minoxidil and oral finasteride 1 mg are the only FDA-approved treatments in males. Minoxidil efficacy is limited, and studies suggest that only one-third of the patients experience a cosmetic benefit or moderate terminal hair regrowth after 1 year of use.[4–7] Scalp irritation, increased seborrhea, erythema and hypertrichosis are commonly reported after topical minoxidil use.[8–10] Oral finasteride 1 mg is FDA-approved for male but not female AGA, due to its teratogenicity. Decreased libido, erectile dysfunction and ejaculatory problems have been reported in around 3–5% of men using finasteride.[11–13] In

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addition, both minoxidil and finasteride require a long-term commitment by the patient on a daily basis, most likely for an individual's life.

Furthermore, in the advanced stages of PHL, the above treatments are less effective and hair transplantation would be the only option for some of these patients. Hair transplantation also comes with limitations. Many balding males have a limited donor supply, which is not sufficient to cover the bald area. Also, many females with PHL have a weak donor area and are not candidates for hair transplantation.[14–16]

The previous observations highlight the importance of finding effective new treatments, which ensure better patient compliance and have limited side effects. Since AGA is characterized by defects in and loss of hair progenitor cells, while hair follicle stem cells (HFSCs) remain viable,[17] transplantation of multipotent stem cells has become a well-accepted treatment option. Autologous cellular micrografts (ACM) is a method that obtains autologous mature stem cells from scalp biopsies of a patient using a preparation system for mechanical disintegration and filtering of solid tissues.

The advocated mechanisms of action of ACM in AGA include the enhancement of hair follicle regeneration by transplantation of mature multipotent stem cells, besides the reactivation of existing stem cells and progenitor cells of miniaturized follicles, by restoring hair growth signaling via the injection of growth factors.[17,18] Yet, these mechanisms have not been established. On the other hand, given the novelty of the treatment method, further data is needed to establish the clinical efficacy in AGA. Yet, the few published reports provide promising results, showing an increase in hair density and thickness in a high percentage of patients.[22,37,38] However, these studies comprise limitations that impede the generalizability of the findings, notably due to limited sample size.

In this paper, we report the short-term clinical efficacy of a single application of ACM in the treatment of male and female AGA. We also proposed a pixel-based method to portray the treatment efficacy and cosmetic benefit in hair loss, in a standardized visual fashion.

Materials and Methods

Design and Setting

A retrospective cohort study was carried out at the author's dermatology clinic in Jeddah, Saudi Arabia, from September 2019 to August 2020.

Population

The study involved 18–65-year-old consecutive males and females with clinically diagnosed and dermoscopy-confirmed AGA, who received a single session of ACM (Regenera Activa®). Only females classified Sinclair 2–4 and males classified Hamilton-Norwood 2–4 were included. Patients using any other topical or systemic medication for hair loss during the 6 months prior to inclusion or during the study period were excluded, as well as those using medical devices such as low-level laser therapy (LLT) or procedures such as platelet-rich plasma (PRP) or micro-needling for hair loss during the same periods. Other exclusion criteria included: cancer within 2 years; pregnancy or breastfeeding; immunosuppression; hemoglobin below 12 mg/dL; presence of other causes of alopecia such as alopecia areata, scarring alopecia or inflammatory scalp disorders; history of hair transplantation; history of scalp tumors; and/or the presence of trichotillomania.

Procedure

Baseline Assessment

As part of our routine practice, patients undergo a structured clinical evaluation to collect demographic and relevant clinical data, followed by dermoscopy to confirm the diagnosis, and global scalp photography following a standardized method and technical settings. Standardized phototrichograms are conducted on all scalps using video-epiluminescence microscopy (FotoFinder Systems, Inc., Columbia, CA, USA) in conjunction with TrichoScan digital image analysis (TrichoScan, Tricolog GmbH, Freiburg, Germany). Trichoscan is a computer-assisted dermoscopy with dedicated software to diagnose the hair loss and to measure its severity.

Autologous Cellular Micrografts (ACM) Procedure

Under local anesthesia, using anesthesia techniques which were previously described by the author,[22] a 2.5 mm punch biopsy was used to extract 3 scalp tissue specimens from the patient's occiput behind the ear, using Rigeneracons medical device (CE certified class I; Human Brain Wave, Turin, Italy). The collected specimens are placed in Rigeneracons by adding 1.5 mL of sterile physiologic solution to the device. The device then generates a cellular suspension by rotation of Rigeneracons at 80 RPM for 2 minutes. Subsequently, the obtained suspension is diluted with an additional 3 mL sterile physiologic solution. The solution was

then injected subdermally into the balding areas of the scalp using a 1mL syringe with a 30-gauge needle; 0.1 mL was injected per point spaced approximately 1 cm apart.

Follow Up and Outcomes

After the intervention, patients were followed up by clinic visits and phone calls for any adverse effects or further concern. The short-term post-treatment assessment including trichometry with TrichoScan digital image analysis was scheduled 1 to 6 months after the intervention, as per the patient's convenience. Trichometry parameters were divided into positive and negative parameters. Positive parameters correspond to parameters whose increase corresponds to favorable evolution (hair growth) and include hair density (N/cm2), average hair shaft thickness (AHST, µm), percent thick hair (%), cumulative hair thickness (CHT), and number of follicular units (%). Negative parameters correspond to parameters whose decrease corresponds to favorable evolution (hair growth) and include percent thin hair (%), yellow dots (N/cm^2), and trichoscopy-derived Sinclair scale (TDSS), which describes the hair midline density calculated from CHT density measured in trichoscopy.[23]

Ethical Considerations

The study was approved by the institutional review board of King Abdulaziz University Hospital in Jeddah (Reference No 235–21). Waiver of consent has been obtained because of the retrospective nature of the study. The confidentiality of the patients' data has been protected and identifiable data of patients have been removed in compliance with the Declaration of Helsinki. The baseline assessments, ACM procedure and follow-up methods described above are part of the routine practice in the dermatology clinic, and were not specifically designed for the present study.

Statistical Methods

Data was managed using Microsoft Excel (version 2017, Microsoft) and statistical analysis was performed with the SPSS version 21.0 for Windows (SPSS Inc., Chicago, IL, USA). For all trichometry parameters, absolute and adjusted relative changes were calculated for each participant as follows:

$$\text{Absolute change(parameter unit)} = x_1 - x_0$$

$$\text{Adjusted relative change}(\%) = 100^*(x_1 - x_0)/X_0$$

Where,

$x_1 - x_0$: pre- to post-intervention change of the parameter value in the given patient,

X_0: baseline population mean of the parameter.

Paired *t*-test was used to analyze the absolute changes in the total study population and in males and females separately. The correlation between pre- and post-intervention values was analyzed using Pearson's correlation.

To visually portray the cosmetic effect of the treatment in the study population, we calculated the area coverage index (ACI) as an estimate of the scalp area, in cm^2, that would be completely covered by 1 cm-long hair, with respect of the hair density and thickness. By assuming a one-µm hair thickness, a 1-cm-long hair would visually cover an area of 1cm × 10^{-4} cm = 10^{-4} cm^2. Thus, by considering the hair density (average number of hairs by cm^2) and thickness (AHST), the ACI was calculated using the following formula:

ACI (cm^{2}hair/cm^{2}scalp) = HD (hair density,N/cm^2) *AHST(µm)* 10^{-4}

Afterwards, a graphical representation was made by generating a 30 × 30 (900-pixel resolution) grid, corresponding to 9cm^2 of the scalp (covered by 9*ACI), comprising filled and blank 1mm^2 square pixels that correspond to the covered and uncovered scalp areas, respectively. The number of filled pixels is proportional to ACI and is calculated as N = resolution*ACI. Filled pixels are dispersed over the grid using a random generator. The ACI definition, calculation formula and pixel transformation method are illustrated in Figure 1.

For each parameter and scalp region, the percentage of participants who had favorable outcome (defined as the desired change occurring within the given parameter) was calculated along with the respective mean absolute change and mean adjusted relative change.

A p value of <0.05 was considered to reject the null hypothesis.

Results

Baseline Demographic and Clinical Characteristics

A total 140 patients were included, 113 (80.7%) of them were female, and mean (SD) age was 32.1 (10.1) years. The

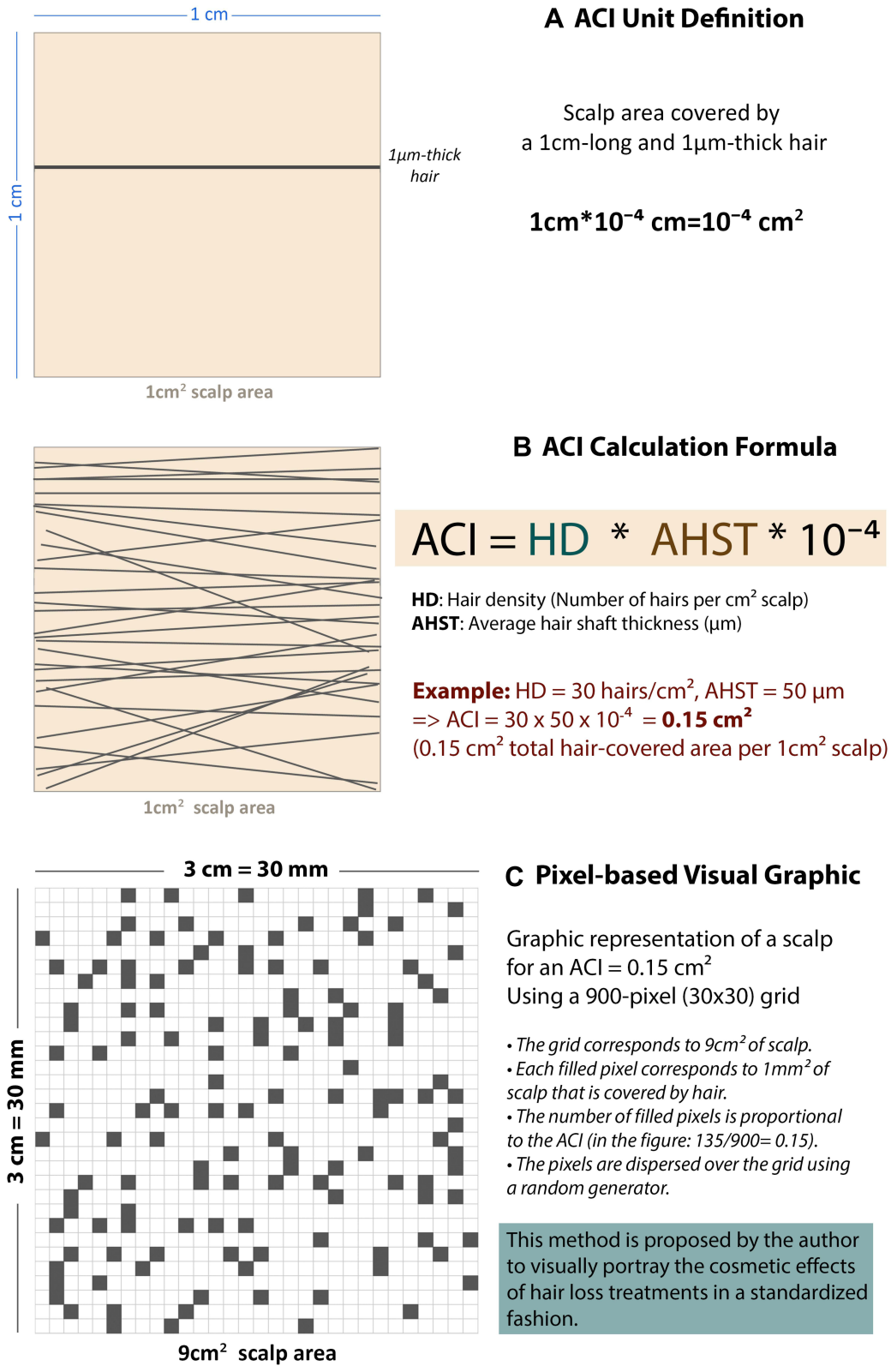

Figure 1 Area coverage index (ACI) definition, calculation formula and pixel transformation method. The figure describes the method proposed by the author to portray the visual aspect of the scalp as a function of the hair density and average hair thickness, by the (**A**) unit definition, (**B**) calculation and (**C**) pixel transformation of an area coverage index (ACI).

Table 1 Pre- and Post-Intervention Trichometry Findings by Scalp Region (Intrasubject Analysis)

Parameter (Unit)	Scalp Region	Baseline		Outcome		R	Mean Absolute Change[a]	p-value
		Mean	SD	Mean	SD			
Positive parameters								
Hair density (N/cm^2)	Frontal	176.87	42.86	182.36	42.49	0.807	5.49	0.015*
	Temporal	134.01	30.48	138.51	29.29	0.752	4.50	0.013*
	Occipital	175.50	38.11	182.62	39.33	0.694	7.12	0.006*
Average hair shaft thickness (μm)	Frontal	48.49	8.14	50.12	9.19	0.740	1.64	0.003*
	Temporal	52.86	9.08	54.74	9.28	0.753	1.88	0.001*
	Occipital	55.58	9.08	56.54	8.93	0.703	0.96	0.102
% thick hair	Frontal	45.71	16.02	48.11	16.98	0.754	2.40	0.016*
	Temporal	54.31	16.18	57.57	15.91	0.715	3.26	0.002*
	Occipital	59.11	16.31	60.85	15.75	0.734	1.74	0.081
Cumulative hair thickness	Frontal	8.50	2.21	9.06	2.63	0.784	0.56	<0.001*
	Temporal	7.05	1.87	7.52	1.76	0.799	0.48	<0.001*
	Occipital	9.65	2.07	10.16	2.12	0.717	0.51	<0.001*
No. follicular unit (%)	Frontal	82.67	12.08	84.31	11.48	0.658	1.64	0.048*
	Temporal	65.17	11.47	66.47	10.98	0.660	1.30	0.099
	Occipital	72.29	12.65	75.06	12.35	0.386	2.77	0.019*
Negative parameters[b]								
% thin hair	Frontal	23.74	11.84	21.93	11.73	0.629	−1.81	0.037*
	Temporal	20.19	11.31	18.87	10.78	0.569	−1.31	0.132
	Occipital	14.04	8.71	13.82	8.34	0.547	−0.21	0.755
Yellow dots (N/cm^2)	Frontal	5.35	8.87	3.42	5.47	0.539	−1.93	0.003*
	Temporal	2.50	4.89	1.43	2.90	0.488	−1.07	0.004*
	Occipital	1.69	3.11	1.41	3.01	0.377	−0.29	0.325
Derived Sinclair grade	Frontal	2.78	0.70	2.62	0.46	0.571	−0.16	0.002*
	Temporal	3.04	0.44	2.93	0.39	0.786	−0.11	<0.001*
	Occipital	2.49	0.37	2.41	0.36	0.724	−0.09	<0.001*

Notes: Test used: Paired *t*-test. *Statistically significant result ($p < 0.05$). [a] Positive results indicate increase, while negative ones indicate decrease in the given parameter. [b] Favorable outcome is indicated by decrease in negative parameters.
Abbreviation: R, Pearson's correlation coefficient.

distribution of males according to Hamilton–Norwood classification showed Class II (48.1%), Class III (18.5%) and Class III Vertex (33.3%). The distribution of females according to Sinclair classification showed Grade 2 (32.7%), Grade 3 (56.6%), and Grade 4 or 5 (10.7%). The median follow-up time was 94 days (range = 39–197 days).

Efficacy of Autologous Cellular Micrografts in the Total Population

Absolute Change

There was increase in all positive parameters including hair density (+4.5 to 7.12 hair/cm^2), AHST (+0.96 to 1.88 μm), % thick hair (+1.74 to 3.26%), CHT (+0.48 to 0.56 unit), and number of follicular units (+1.30 to 2.77), depending on the scalp region, and the improvements were more remarkable in frontal region where all these changes were statistically significant ($p < 0.05$). Additionally, negative parameters decreased, notably in frontal region in % thin hair (−1.81%, $p = 0.037$) and yellow dots (−1.93 N/cm^2, $p = 0.003$). In the majority of parameters, pre- and post-intervention values were moderately to strongly correlated (R = 0.539–0.807) (Table 1).

Adjusted Relative Change

Depending on scalp region, there was relative increase in hair density (up to 4.1%), AHST (up to +3.6%), % thick hair (up to +6.0%), CHT (up to +6.7%), and number of

follicular units (up to +3.8%), with relative decrease in % thin hair (down to −7.6%), as adjusted to the population's baseline. Besides, a substantial relative decrease in yellow dot density was observed, notably in frontal (−36.0%) and temporal (−42.9%) regions. This was associated with decline in TDSS (Figure 2).

ACI and the Cosmetic Effect

The mean hair-covered scalp area in the total population increased in all three scalp regions including frontal (from 0.85 to 0.91 cm^2 hair/cm^2 scalp), temporal (0.70 to 0.75 cm^2 hair/cm^2 scalp) and occipital (0.96 to 1.02 cm^2 hair/cm^2 scalp), and all results were statistically significant ($p < 0.001$). Results were depicted in a randomly generated 900-pixel grid representing $9cm^2$ of the scalp (Figure 3).

Effect Size in Favorable Responders

Two-thirds of patients (63.6–66.4%) had a favorable change in CHT, with a mean absolute change 1.2–1.4 unit depending on the scalp region. Further, more than 50% had favorable change in the other parameters, except yellow dots that decreased in up to 43.6% of the participants depending on the scalp region (Table 2).

Efficacy by Gender

In Females

The mean CHT increased by 0.52 to 0.57 units, associated with a significant increase in both AHST (+1.41 to 2.17μm) and % thick hair (+2.64% to 4.16%) in all 3 scalp regions. Increase in hair density was not statistically significant in the frontal region. Further, all negative parameters decreased, but statistical significance was not consistent throughout scalp regions (Table 3). The pre and post treatment scalp photographs of a sample female patient are depicted in Figure 4-Patient 1.

In Males

The frontal region showed a statistically significant increase in mean CHT (+0.66 units, $p = 0.045$) associated with a remarkable increase in mean hair density (+11.2 hair/cm^2, $p = 0.031$) in reference to baseline (Table 3). The pre and post ACM scalp photographs of a sample male patient are depicted in Figure 4-Patient 2.

Discussion

Summary of Findings

The present study provides the largest series of AGA patients treated with ACM to date. Pre- to post-intervention assessment showed favorable changes in all positive and negative trichometry parameters, including growth of an average 5–7 new hairs per cm^2 of scalp and an increase in the average hair thickness by 1.6–1.9μm, which is reflected by an increase in the percentage of thick hairs and proportional decrease in the percentage of thin hairs. Additionally, there was considerable reduction of yellow dots by 1–2 per cm^2 of scalp combined with increase in the percentage of follicular units by up to 3%. All these changes resulted in the enhancement of the CHT by an average 0.5–0.6 units, representing 5–6 mm^2 increment in hair coverage index per cm^2 of scalp, which induced a significant cosmetic effect as demonstrated by the visual model developed by the author in this study. Such favorable outcomes were observed in up to two-thirds of patients, depending on the parameter, and the magnitude of changes among positive responders was more remarkable, notably in the frontal region. These findings are very encouraging and indicate multiple effects of ACM in improving the hair of AGA patients in the short term.

Pathophysiological Basis of ACM Mechanisms of Action

Mature HFSCs, located in the hair follicle bulge, are multipotent cells that play a key role in the regeneration of hair follicles and other scalp skin structures. They have the ability to self-regenerate between the telogen and anagen phases of the hair cycle; or migrate down the hair matrix and become progenitor cells, forming the internal hair follicles and hair stem. The activation of bulge-located stem cells is controlled by the surrounding micro-environment "niche" including their daughter cells, as well as by dermal papilla cells (DPCs) signaling pathways, notably the Wnt/β-catenin pathway which is crucial for entry into the anagen phase. DPCs further contribute in regulating hair growth by secreting hair growth-stimulating factors such as insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor, and vascular endothelial growth factor, which respectively have an autocrine effect on the dermal papilla itself and paracrine effect on hair follicle epithelial cells.[19,24–30]

The most supported pathophysiological mechanism of AGA involves a suppressive effect of systemic or locally produced (by balding DPCs) dihydrotesterone on follicular keratinocyte growth, which leads to catagen. This effect is mediated by the upregulation of transforming growth

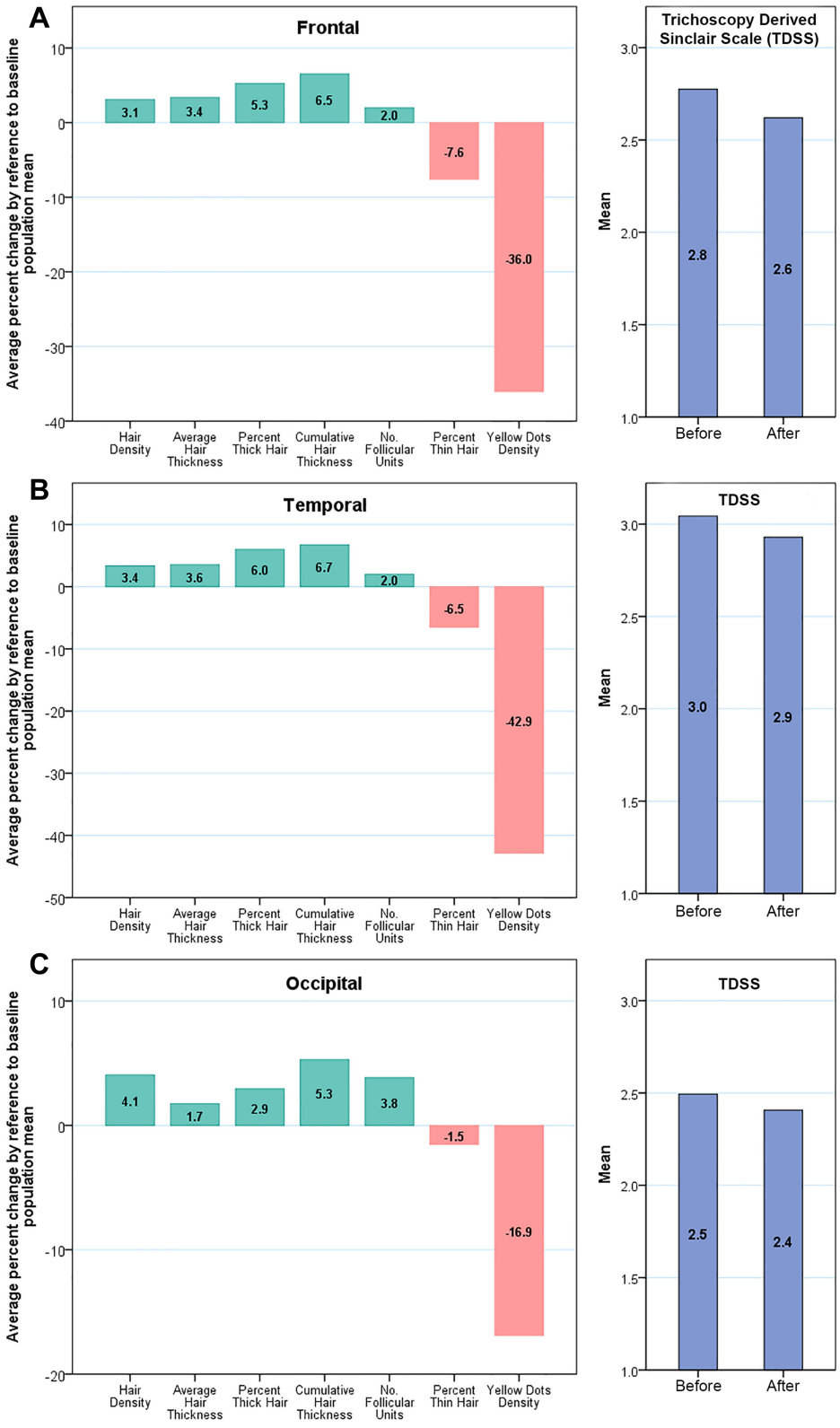

Figure 2 Change in key indicators of hair growth, by scalp region, in male and female androgenic alopecia after treatment with autologous cellular micrografts (ACM). Pictures depict: 1) relative pre- to post-intervention change (in percent, left panels) of the 5 positive (green bars) and 2 negative (Orange bars) parameters as adjusted by the respective baseline population mean, that is, bar heights represent the percentage of change in the given parameter by reference to the baseline population mean; 2) mean derived Sinclair grade (right panels) in baseline versus after treatment with ACM. Panels correspond to findings in different scalp regions including frontal (**A**), temporal (**B**), and occipital (**C**) regions.

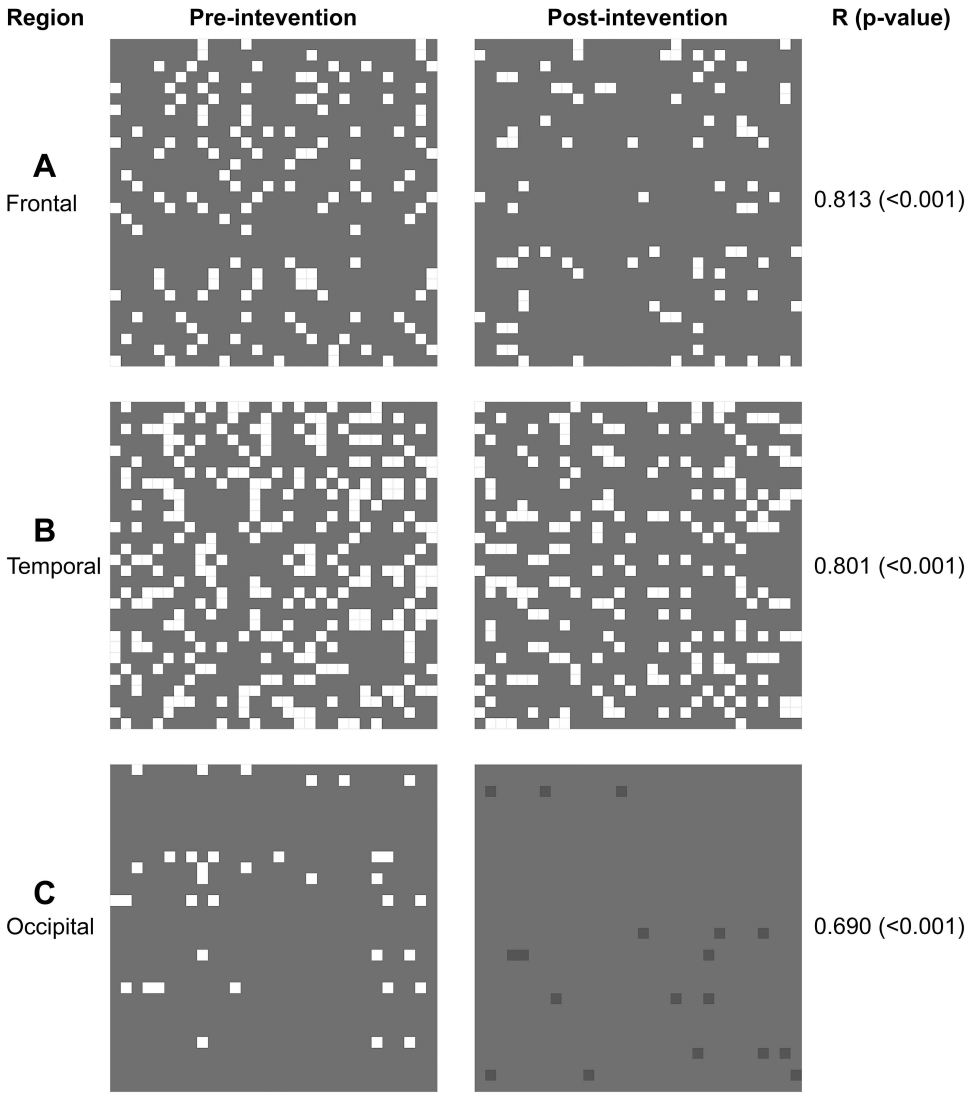

Figure 3 Short-term hair growth in male and female androgenic alopecia after treatment with autologous cellular micrografts (ACM). Pictures depict pre- to post-intervention change in area coverage index (ACI) in the frontal (**A**), temporal (**B**), and occipital (**C**) regions. Each grid represents 9cm^2 (3×3cm) of scalp area; where gray cells represent the scalp area covered by hair, white cells represent the scalp areas not covered by hair, and dark gray cells represent highly dense areas indicating ACI > 1cm^2.

factor beta (TGF-β) and Wnt antagonist DKK-1 production by DPCs. These alterations result in a number of pathohistological anomalies including impaired HFSC differentiation, oxidative stress and perifollicular fibrosis and inflammation, which altogether lead to the chronic hair miniaturization resulting in smaller dermal papillae and a reduction in CD200$^+$ progenitor cells.[31–37] On the other hand, although progenitor cells are damaged in AGA, the HFSCs remain preserved, which explains the reversibility of the condition. These observations constitute the foundation for ACM, which consist of the transplantation of mature multipotent stem cells in the balding areas, thereby enabling hair follicle regeneration.[17] Besides unaffected areas of the scalp, ACM may use multipotent stem cells originating from adipose tissue.[38] Another plausible mechanism of action of ACM is the reactivation of already existing stem cells and progenitor cells of miniaturized follicles, by reinstating the hair growth signaling via the injection of growth factors.[18]

Reports on Efficacy of ACM in AGA

The few available reports on ACM efficacy are encouraging. In a small placebo-controlled trial (N = 11), the use of ACM showed ~29% relative increase in hair density in treated scalp areas, approximately 6 months after treatment.[19] Another study including 17 patients showed

Table 2 Change in Hair Growth Indicators Among Favorable Responders

Parameter (Unit)	Scalp Region	Favorable Response Rate, n (%)	Mean Absolute Change (Unit)[a]	Adj. Mean Relative Change (%)[a]
Positive parameters				
Hair density (N/cm^2)	Frontal Temporal Occipital	85 (60.7) 86 (61.4) 79 (56.4)	21.8 17.6 26.7	12.3 13.1 15.2
Average hair shaft thickness (μm)	Frontal Temporal Occipital	76 (54.3) 79 (56.4) 76 (54.3)	6.2 6.2 5.6	12.8 11.7 10.1
% thick hair	Frontal Temporal Occipital	78 (55.7) 85 (60.7) 74 (52.9)	10.6 10.7 10.0	23.2 19.7 16.9
Cumulative hair thickness	Frontal Temporal Occipital	93 (66.4) 90 (64.3) 89 (63.6)	1.4 1.2 1.4	16.4 16.3 14.6
No. follicular unit (%)	Frontal Temporal Occipital	69 (49.3) 79 (56.4) 73 (52.1)	9.3 7.6 12.0	11.2 11.7 16.6
Negative parameters[b]				
% thin hair	Frontal Temporal Occipital	75 (53.6) 77 (55.0) 62 (44.3)	−8.8 −8.4 −7.0	−37.1 −41.6 −49.9
Yellow dots (N/cm^2)	Frontal Temporal Occipital	61 (43.6) 49 (35.0) 41 (29.3)	−7.1 −4.8 −3.5	−132.7 −192.0 −207.1
Derived Sinclair grade	Frontal Temporal Occipital	83 (59.3) 80 (57.1) 75 (53.6)	−0.4 −0.3 −0.3	−14.4 −9.9 −12.0

Notes: [a]Positive results indicate increase, while negative ones indicate decrease in the given parameter. [b]Favorable outcome is indicated by decrease in negative parameters.

patient-perceived increase in hair thickness (12/17 patients) and reduction in hair fall (8/17), while the remaining cases observed no change for either parameter.[20] More recently, Ruiz et al studied the efficacy of ACM at 4, 6 and 12 months, among 100 AGA and showed significant increase in hair density (+33.3/cm^2) and percentage of thick hair (+5.6%) at 2 months.[21]

Efficacy of ACM by Gender

Findings from the present study suggest a gender-specific effect of ACM in AGA. The most remarkable effect in males was the increase in hair density, while in females it was the promotion of hair thickness and reduction of the number of yellow dots. Interestingly, the two patterns resulted in a comparable improvement of CHT across genders. Additionally, although the occipital area was not treated in any of the study patients, women experienced significant improvement in all hair growth indicators in the occipital region with a single ACM session, which could have an implication in improving the donor area in females requiring a hair transplantation.

Pixel-Based Representation of Cosmetic Effect

The author proposes the use of a pixel-based graphical method to portray the cosmetic effects of treatments in hair loss in a standardized fashion. This method provides an intuitive visualization of the change in scalp area coverage

Table 3 Hair Growth Indicators by Gender and Scalp Region in Male and Female Androgenic Alopecia After Treatment with Autologous Cellular Micrografts (ACM)

Parameter (Unit)	Scalp Region	Female (N = 113)		Male (N = 27)	
		Mean Absolute Change[a]	p-value	Mean Absolute Change[a]	p-value
Positive parameters					
Hair density (N/cm^2)	Frontal	+4.12	0.103	+11.22	0.031*
	Temporal	+5.75	0.003*	−0.74	0.869
	Occipital	+6.43	0.017*	+10.00	0.192
Average hair shaft thickness (μm)	Frontal	+1.84	0.002*	+0.78	0.549
	Temporal	+2.17	001*	+0.67	0.570
	Occipital	+1.41	0.028*	−0.89	0.549
% thick hair	Frontal	+2.84	0.005*	+0.56	0.850
	Temporal	+4.16	0.001*	−0.52	0.754
	Occipital	+2.64	0.016*	−2.04	0.398
Cumulative hair thickness	Frontal	+0.53	0.001*	+0.66	0.045*
	Temporal	+0.57	<0.001*	+0.06	0.817
	Occipital	+0.52	<0.001*	+0.45	0.244
No. follicular unit (%)	Frontal	+1.21	0.189	+3.44	0.080
	Temporal	+1.81	0.039*	−0.81	0.661
	Occipital	+3.35	0.010*	+0.33	0.907
Negative parameters[b]					
% thin hair	Frontal	−2.16	0.030*	−0.33	0.849
	Temporal	−1.81	0.079	+0.74	0.605
	Occipital	−0.73	0.339	+1.96	0.208
Yellow dots (N/cm^2)	Frontal	−2.15	0.006*	−1.00	0.159
	Temporal	−1.20	0.007*	−0.52	0.283
	Occipital	−0.17	0.618	−0.78	0.146
Derived Sinclair grade	Frontal	−0.16	0.007*	−0.13	0.037*
	Temporal	−0.14	<0.001*	−0.01	0.778
	Occipital	−0.09	<0.001*	−0.06	0.269

Notes: Test used: paired *t*-test. [a]Positive results indicate increase, while negative ones indicate decrease in the given parameter. [b]Favorable outcome is indicated by decrease in negative parameters. *Statistically significant result ($p < 0.05$).

after treatment. It can be used in all types of hair loss and treatments (transplantation, topical treatments, etc.) and can have interesting clinical and research applications.

Limitations

The external validity of the present study may be limited by the retrospective and noncontrolled design. Further, the present design does not demonstrate the final clinical improvement due to the short-term endpoint of the study outcomes. A longer follow-up study will be needed in the future.

Conclusion

Two-thirds of patients with AGA would respond favorably to a single treatment session with ACM in the first 6 months following treatment. The pre- to post-ACM trichometry analysis showed significant improvement in hair regrowth, hair thickening, promotion of follicular units and reduction of yellow dots; all combined, these effects result in an increase in the hair area coverage index, representing a noticeable cosmetic change. There is a probable gender-specific effect of ACM in AGA that should be further studied.

Figure 4 Pre and post treatment scalp photographs of a sample female and male patients with androgenetic alopecia, before and after treatment with autologous cellular micrografts (ACM). Photographs show cosmetic improvement after autologous cellular micrografts in a female (1) and a male (2) patient. In the female patient (1), there was increase in CHT from 10.3, 7.5 and 9.9 to 10.5, 8.3 and 12 mm/cm^2 in frontal, temporal and occipital areas, respectively. In the male patient (2), there was increase in CHT from 12.7 to 13.2 mm/cm^2 in the frontal and 8.8 to 12.3 mm/cm^2 in the occipital area, while CHT decreased from 8.5 to 7.5 mm/cm^2 in the temporal area.

Abbreviations

ACI, (Scalp) area coverage index; ACM, autologous cellular micrografts; AGA, androgenetic alopecia; AHST, average hair shaft thickness; ANOVA, analysis of variance; CHT, cumulative hair thickness; DPC, dermal papilla cells; FDA, Food and Drug Administration; HFSC, hair follicle stem cell; LLT, low-level laser therapy; PHL, pattern hair loss; TGF-β, transforming growth factor beta.

Data Sharing Statement

The database that supports the findings of the present study is available upon written request from the author.

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Disclosure

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Scientific Research

Adipose Derived Stem Cells and Growth Factors Applied on Hair Transplantation. Follow-Up of Clinical Outcome

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Abstract

Different studies show the need of immature adipose cell to induce the proliferation of bulge stem cells in order to kick off the anagen phase of hair cycle. Furthermore, the adipose derived stem cell, adipose progenitors, and growth factors secreted by mature adipocytes can help the wound healing and the vascular neogenesis. Nowadays, it is not known any protocol of tissue regeneration applied to hair transplantation, especially if aimed to the reconstruction of the main vascular network for the engraftment of transplanted hair and the healing process. The aim of the work is to investigate how the application of autologous cellular suspension obtained by Rigenera system, mechanical fragmentation procedure which allows to obtain a physiological saline solution consisting of a heterogeneous pool of cells rich in adipose derived mesenchymalstem cells and growth factors, helps the wound healing and engraftment of the transplanted hair. During hair restoration surgery, the adipose tissue recovered from the discard of follicular slicing, was processed using the Rigenera system. The obtained cell suspension was applied in the area of hair transplantation, increasing the natural background of adipocyte lineage and raising the amount of growth factors. In addition, the cellular suspension was applied to the suture on the occipital region. The cell population was characterized by FACS. The monthly evaluation of hair transplantation follow-up with photos and the patient's impressions demonstrates that there is a faster healing of the micro-wound and a continuous growth of the transplanted hair even two months after the procedure, with a shortening of the dormant phase. In conclusion, this new approach aims to integrate regenerative medicine and hair restoration surgery in order to improve the outcome for the patient. It would be wonderful to continue this research to elaborate on the molecular cause behind this satisfying clinical.

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Keywords

Stem Cell, ADSC, Hair Transplantations, Regenerative Medicine, Hypoderm Hair Transplantations

1. Background

The field of hair transplantations has made countless step forward and now it is possible to obtain results not even imagine thirsty years ago.

In the last ten years, we have witnessed the rise of regenerative medicine applied at many surgical disciplines aiming to increase the results and reduce the pain of patients. Nowadays, except for Platelet Rich Plasma (PRP), it is not known any protocol of tissue regeneration applied to hair transplantation, especially if aimed to the reconstruction of the main vascular network for the engraftment of transplanted hair and the healing process.

A number of researches underline the existence of cells in the adult body capable of repairing and regenerating damaged tissues.

Adipose tissue is a multifunctional organ that contains various cellular types, such as mature adipocytes and the stromal vascular fractions (SVF), which consists of endothelial cells, pericytes, fibroblasts, pre-adipocytes and mesenchymal stem cells, called Adipose Derived Stem Cells (ADSC) (**Figure 1**). These pluripotent cells with their secretome mediate different skin regenerative effects, such as wound healing, antioxidant protection and antiwrinkling [1]. Autologous ADSC have been applied for several regenerative treatments such as widespread traumatic calvarial bone defects [2], breast augmentations [3], fistulas in patients with Crohn's disease [4] and for wound healing in treatment of chronic ulcers [2].

Festa [5] shows the need for immature adipocytes to promote the proliferation of bulge stem cells. Sumikawa [6] shows the potential of leptine and adipokine as an inducer of anagen phase. Furthermore, growth factors stimulate hair growth in both *ex vivo* and *in vivo* animal model [7] [8].

It is clear that the adipocyte lineage is critical to tissue regeneration and hair growth.

2. Aim

The aim of this work is to evaluate how a pool of cells consisting of ADSC, pericytes, endoteliocytes, preadipocytes and their secretome can improve the hair transplantation outcome, helping wound healing and follicular units' engraftment.

3. Methods

3.1. Subjects

Three patients, subjected to hair restoration surgery with the application of adipose derived stem cells and growth factors, were monitored after 5 days, 2 weeks and 1 month. The selection of the patients was casual and based on their availability.

3.2. Cellular Suspension Obtainment

During hair restoration surgery, a strip of scalp form the occipital region is cut and sliced to isolate the follicular units. Below the dermis there are hypodermis and adipose tissue that had been discarded in the past. These tissues were processed using Rigenera system.

Rigenera device is a safe standardized sample preparation system, for the automated mechanical disaggregation of cells population. This mechanical fragmentation, allows extracting from tissue only smallest cells that represents the progenitor cells responsible of the tissue formation. In addition, this system is able to cut, without crash, the single extracellular matrix constituents, which play an important role in reducing inflammatory process and so help the healing of tissue. **Figure 2** states all steps to obtain a cell suspension.

4. FACS

FACS analysis was performed to evaluate the quantity and quality of cell suspension from two different patients.

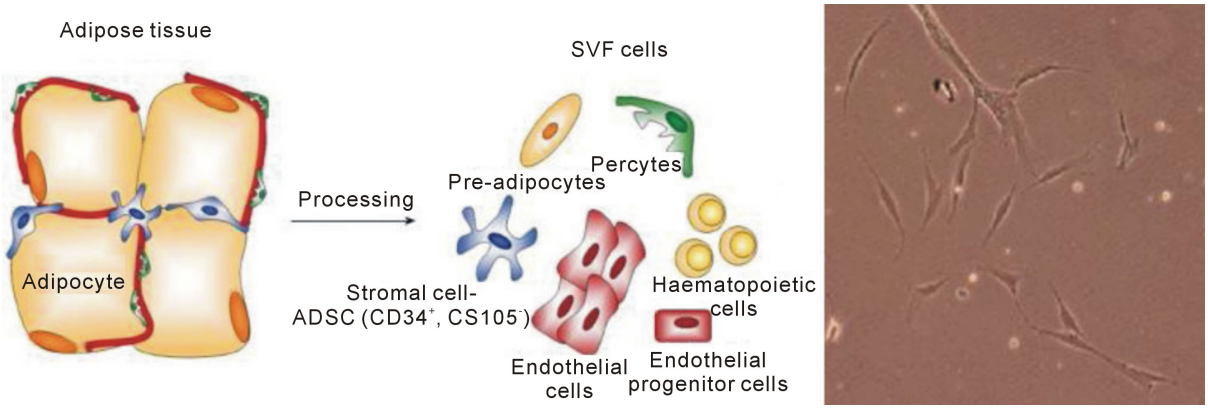


Figure 1. Adipose tissue and photo of ADCS by optical microscope.

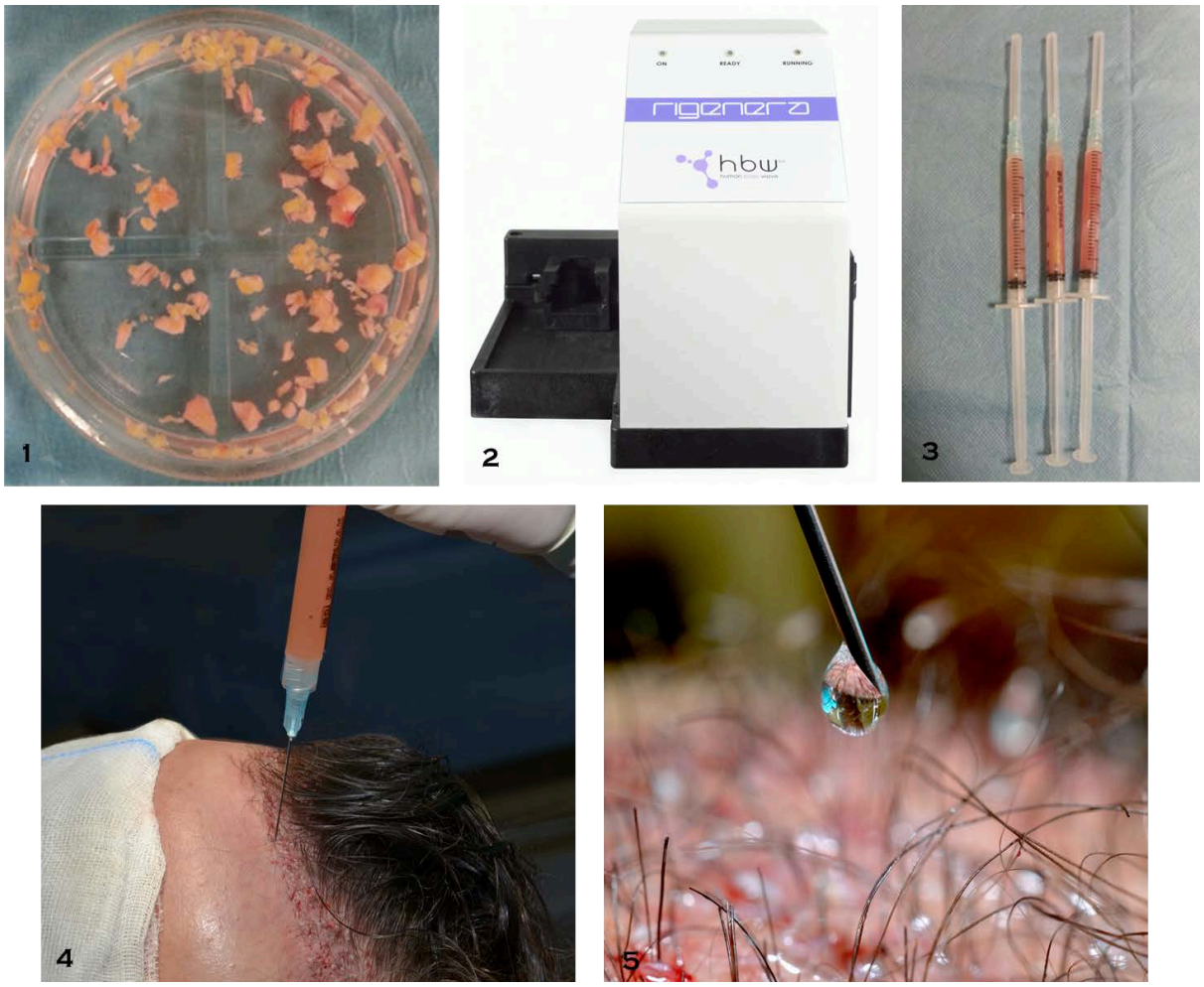


Figure 2. Rigenera System: 1) Hypo-derma and adipose tissue from the occipital region; 2) Rigenera system: a piece of tissue +1 ml of saline solution was inserted in Rigeneracons (CE/EC disposable medical devices containing a stainless steel grid with 100 hexagonal holes of 50 µ each surrounded by six micro blades) and mechanically fragmented with Rigenera for 3 min. The cells passed through the grid into the liquid suspension; 3) Cell suspension was collected with a syringe. The disaggregation and filtration results in a physiological saline solution consisting of heterogeneous pool of cells and growth factors; 4) Cell suspension was injected subcutaneously; and 5) Dropped on the micro incisions made for the engraftments of hair. The suspension was applied before and after the hair insertion. (For some patients the suspension was applied also on the donor area wound)

The gating was made for CD146 and CD34, typical of adipose derived mesenchymal stem cells. The cells were also observed at optical microscope.

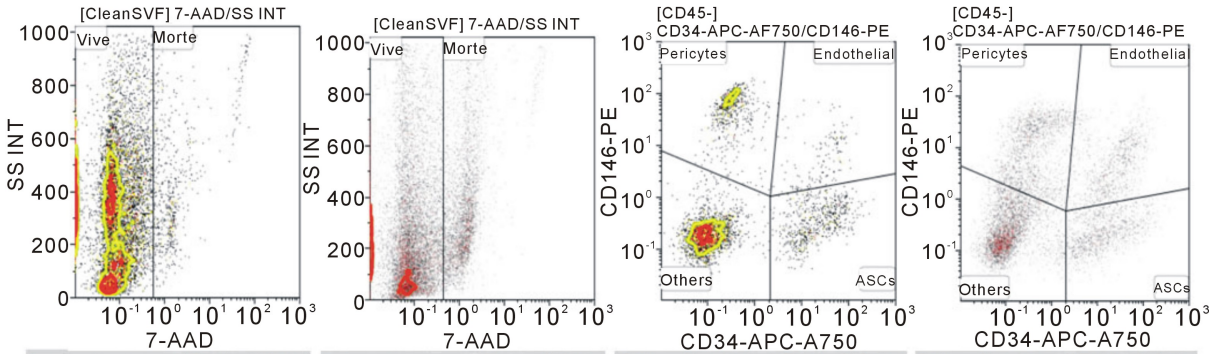
5. Results

5.1. Cell Suspension Analysis

The cellular vitality is 93% for the 1st patients and 74% for the 2nd. This represents a good result especially after mechanical fragmentation.

Both gating for CD146 and CD34 shows that the cellular suspension obtained by Rigenera is a heterogeneous pool of cells composed by erythrocytes, epithelial cells, ADSC and 90% of living cells, which correspond to immature adipocytes and ADSC at the first differentiation stage (**Figure 3**).

The cells are in the active phase of the cell cycle; lots of them are doubling and splitting, showing that Rigenera sorting allows the collection of young and active cells, discarding the quiescent and old cells at the end of their functional utility (**Figure 4**).



Gate	Number	%Total	%Gated
All	9529	20.80	100.00
E3	0	0.00	0.00
E4	0	0.00	0.00
Morte	642	1.40	6.74
Vive	8887	19.40	93.26

Gate	Number	%Total	%Gated
All	15,704	23.00	100.00
E3	0	0.00	0.00
E4	0	0.00	0.00
Morte	4052	5.93	25.80
Vive	11,652	17.06	74.20

Gate	Number	%Total	%Gated
All	3887	8.48	100.00
E3	646	1.41	16.62
E4	140	0.31	3.60
Morte	2417	5.28	62.18
Vive	684	1.49	17.60

Gate	Number	%Total	%Gated
All	8759	12.83	100.00
E3	1396	2.04	15.94
E4	1179	1.73	13.46
Morte	4138	6.06	47.24
Vive	2046	3.00	23.36

Figure 3. FACS made on a pool of cells obtained from the mechanical fragmentation of the hypodermic and adipose tissue from the occipital region of two patients. 1) Vitality of cells; 2) Gating for CD146 and CD34 in order to underline the presence of Adipose Derived mesenchymal Stem Cells (ADSC).

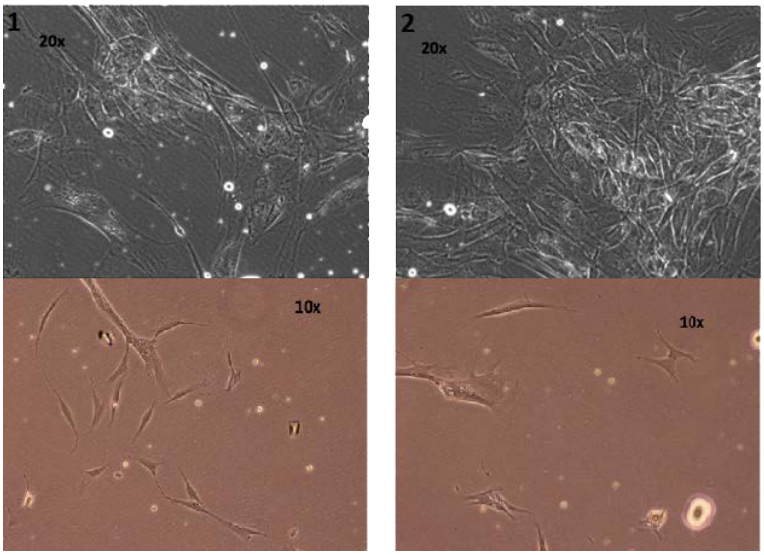


Figure 4. Photos of cellular suspension of the two patients by optical microscope.

5.2. Patient's Follow-Up

Only two weeks after transplantation the healing of micro wounds was complete. Hair continued growing, greatly improving the patient's mood and self-confidence. Also the wound on the donor area was perfectly healed (**Figure 5**).

Five days after hair restoration surgery, the patient reported a perfect recovery, without any swelling and edema. The small scabs were clean and dry, ready to fall (**Figure 6**).

One month after surgery, a maintenance of transplanted hair and a perfect healing of micro wounds are visible (**Figure 7**).

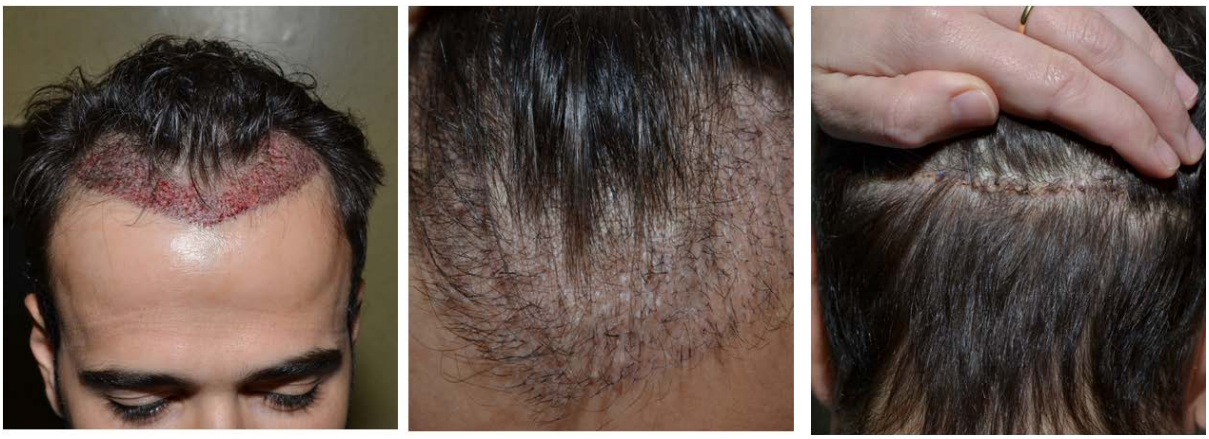
5.3. Patient's Impressions

For all the patients the pain was very mild and for one of them was completely absent.

They also reported a reduction of post operatory edema and swelling. The perception of pain was established by VAS (Visual Analogic Scale) (**Figure 8**).

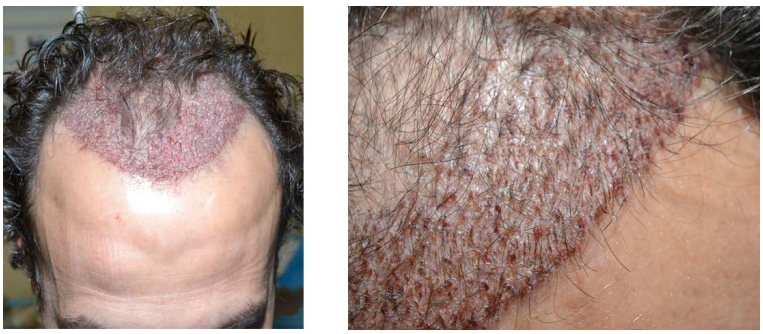
6. Discussion

Rigenera system gives the possibility to extract from tissue only the smallest cells that represent the progenitors responsible for the tissue formation. It allows to cut, without crash, the single extracellular matrix constituents, which are important in reducing the inflammatory process and so to help the healing of tissue. Furthermore, it crashes mature adipocytes freeing up many growth factors. In this way it is possible to maintain a sort of "cellular-niche" in which every cell and growth factor plays its role in tissue regeneration.



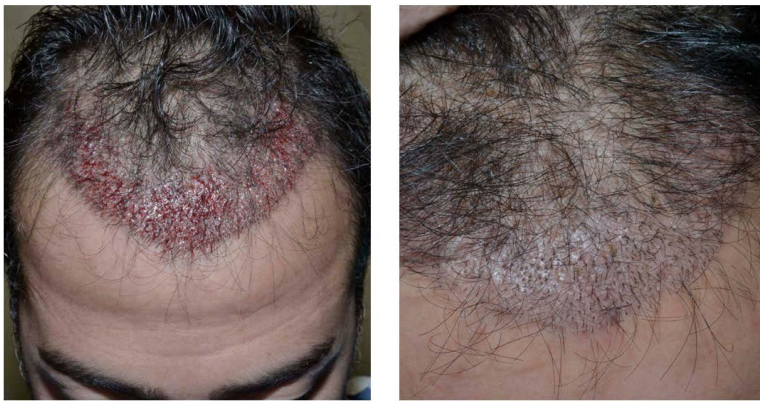
(a) (b) (c)

Figure 5. Patient 1. (a) Immediately after the hair restoration surgery; (b) and (c) two weeks after.



(a) (b)

Figure 6. Patient 2. (a) Immediately after the hair restoration surgery; (b) Five days after.



(a) (b)

Figure 7. Patient 3. (a) Immediately after the hair restoration surgery; (b) one month after.

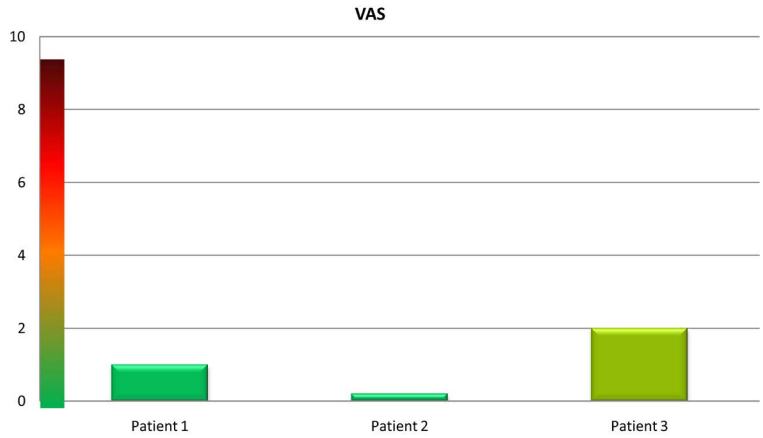


Figure 8. Visual analogic scale. The VAS shows the pain suffered by the patients: From 0 to 2 the pain was VERY MILD, from 2 to 4 MILD, from 4 to 6 MEDIUM, from 6 to 8 STRONG, from 8 to 10 VERY STRONG.

Applying these cells in the area of hair transplantation increases the natural background of adipocyte lineage, which is already present in the bulge and dermal papilla region. It raises the amount of growth factors easing the healing process and helping hair growth and engraftment of transplanted hair. It would be wonderful to continue this research to elaborate on the molecular cause behind these satisfying clinical results and to carry out a bigger and more complete clinical trial.

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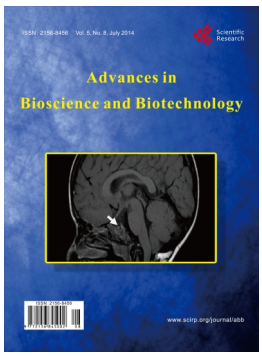

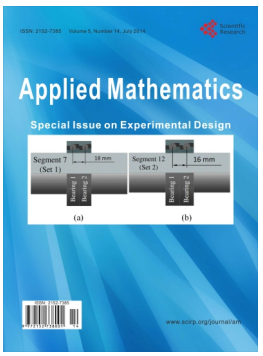

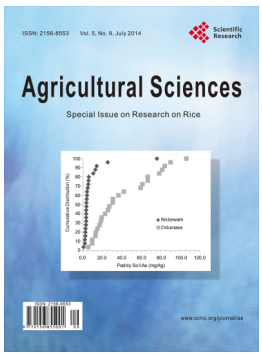

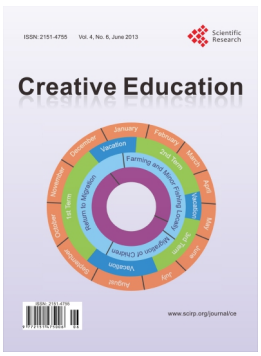

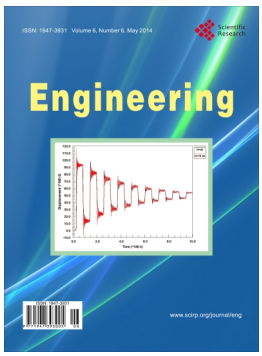

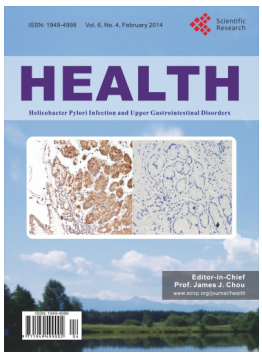

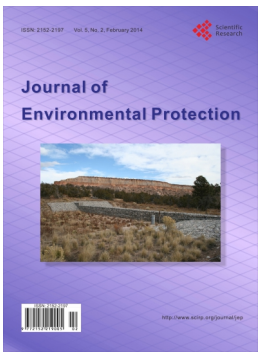

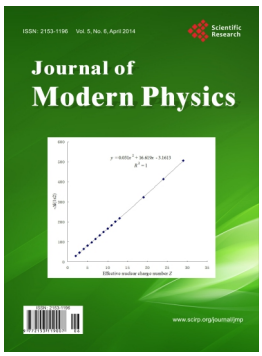

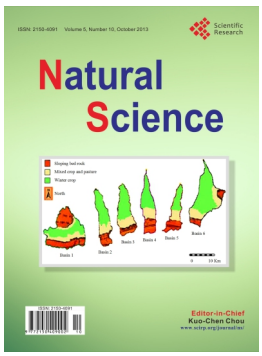

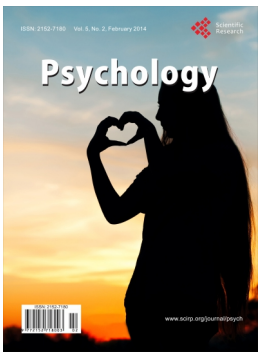

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Microscopic and Histologic Evaluation of the Regenera® Method for the Treatment of Androgenetic Alopecia in a Small Number of Cases

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ABSTRACT

Alopecia is a generalized problem concerning a large sector of the population, both men and women. Some of the most common treatment options for androgenic alopecia are a useful, though the results obtained are not always successful. Apart from known medical treatments, there are other possibilities, like regenerative medicine procedures. The goal of this paper is to assess changes occurring on the scalp, hair bulbs and hairs after applying an autologous suspension obtained using the Rigenera® system.

Keywords: *Alopecia, AGA, regenerative medicine, Rigenera.*

INTRODUCTION

Alopecia is defined as any type of pathological hair loss anywhere on the skin surface.[1] It is a generalized problem concerning a large sector of the population, both men and women, especially when located on the scalp. The wide range of products and treatments against hair issues reflects their high demand and the incidence of this problem.[1] Alopecia can be classified in two main groups: scarring alopecia and non-scarring alopecia (Table I). The main difference is that in non-scarring alopecia, hair follicles are not destroyed, whereas scarring alopecia[2] involves their destruction. The most frequent type is androgenetic alopecia (AGA), also known as "common baldness". It occurs when the hormone 5-α-dihydrotestosterone (5-α-DHT) acts on predisposed hair follicles of the scalp, gradually miniaturizing them until they disappear.[3]

Table1. *Classification of Alopecia.*

Scarring	Non-Scarring
Infectious	Androgenetic (AGA)
Physical-chemical agents	Areata
Neoplasias	Traumatic
Dermatosis	Drugs
Hereditary diseases	Systemic diseases
	Hereditary syndromes

The most common treatment options for AGA are described in Table II. Topical 2% and 5% Minoxidil is a useful treatment both for men and women.[4] However, results obtained are not always successful. This is due to: i) inefficacy of the drug; ii) occurrence of side effects, like pruritus, desquamation or headache; iii) failure to continue performing the treatment; and iv) withdrawal from the treatment due to patients discomfort, who complain about their hair looking greasy or dirty after application.

Finasteride and, more recently, Dutasteride have been approved to treat male pattern AGA.[2] Orally administered at 1 mg per day, these drugs act by inhibiting the 5-α-reductase enzyme type 2 in the case of Finasteride, or as inhibitors of 5-α-reductase enzymes types 1 and 2 for Dutasteride. Thus, they block the conversion of free testosterone to 5-α-DHT, reducing its direct action on hair follicles.[3] The need of a daily schedule for a long time causes high rejection from the patients, who often withdraw from the treatment. Women have an alternative, which is also orally administered: antiandrogens, like spironolactone and cyproterone acetate.[3] However, due to their interrelation with other

hormones and changes in hormonal balance that women suffer throughout their lives, it is not possible to prescribe standardized doses or a simple action protocol.

On the other hand, there are other different treatments. Platelet rich plasma (PRP), for instance, involves a high economic cost for the patient, provides different results and lacks standardized doses.[4] With capillary surgery, hair follicle transplants are performed using the FUE (Follicular Unit Extraction) or FUSS (Follicular Unit Strip Surgery) techniques, and as long as they are properly indicated and carried out, they yield excellent results in male patients. In female pattern AGA (FAGA), hair transplant is not usually a good therapeutic option.

Table2. *Classification of medical treatments for alopecia.*

Topical	Oral Drugs	Injectable Drugs	Fototherapy	Surgery
Minoxidil	Finasteride	Cortisone	Laser	FUE
Antimycotics	Dutasteride	PRP	LED	FUSS
	Corticosteroids			
	Antiandrogens			

Apart from known medical treatments, there are other possibilities with less solid scientific foundation, but that nonetheless are highly accepted by the population: oral or injectable multivitamin supplements, shampoos, lotions, masks, homeopathy and phytotherapy, among others. Very few of these help to stop hair loss or are really effective to treat alopecia.[3]

Results obtained with regenerative medicine procedures for the treatment of complex injuries,[5] regeneration of soft tissues[6] and bone regeneration,[7] have attracted a lot of interest in their application for several pathologies or hair conditions.

The goal of this paper is to objectively evaluate a very small number of patients with AGA to assess changes occurring on the scalp, hair bulbs and hairs after applying an autologous suspension obtained using the Rigenera® system.

METHODS

The treatment consists of the mechanical disintegration of a sample of tissue obtained by a skin punch and subsequent filtration (50 microns) to be intradermally administered in the affected area according to technical specifications (Regenera® Protocol, Rigenera® System, Human Brain Wave SRL, Turin).

For this descriptive study, 3 patient volunteers were consecutively and randomly recruited. The defined inclusion criteria were: males over 18 years old diagnosed with male pattern AGA. Exclusion criteria included: allergy to lidocaine, healing issues, scarring alopecia, chronic drug treatment, oncologic processes and having performed any hair loss treatment —topical, oral or injectable— between 3 months prior and 3 months after the date of applying treatment — except taking vitamin supplements and applying topical lotions or shampoos.

The suspension was applied on the scalp using mesotherapy, and the variables assessed were:

- Hair thickness: Measured with a micrometer (MDC-1"MX Mitutoyo® model, Mitutoyo Corporation™)
- Hair Loss Test: At control visits, 30 days after treatment, involving counting hairs over a white towel after combing it forward for 60 seconds
- Biopsy[9-13] for immunohistochemical stains: Ki-67 for the identification of cells found in the proliferative phase of the cell cycle, CD34 for the location of the vascular endothelium and Vimentin[11] for the location of an intermediate filament indicating an increase in fibroblasts and perifollicular collagen.

Follicular units and biopsies were taken from the upper parietal region of the scalp.

RESULTS

Three male patients were treated with the Rigenera® system. According to the scale of Hamilton, two had alopecia type III, and one had alopecia type IV. The results of the micrometer measurements are summarized in Table 3.

Table3. *Measurements with micrometer.*

Patient 1	Hair 1 (Mm) Total (Proximal - Intermediate – Distal)	Hair 2 (Mm) Total (Proximal - Intermediate – Distal)	Hair 3 (Mm) Total (Proximal - Intermediate – Distal)
Baseline	0.186 (0.058 - 0.074 - 0.054)	0.152 (0.054 - 0.050 - 0.048)	0.15 (0.052 - 0.051 - 0.047)
1 Month	0.185 (0.065 - 0.065 - 0.055)	0.187 (0.064 - 0.072 - 0.051)	0.179 (0.063 - 0.062 - 0.054)

Patient2	Hair 1 (Mm) Total (Proximal - Intermediate – Distal)	Hair 2 (Mm) Total (Proximal - Intermediate – Distal)	Hair 3 (Mm) Total (Proximal - Intermediate – Distal)
Baseline	0.183 (0.062 - 0.062 - 0.059)	0.181 (0.060 - 0.060 - 0.061)	0.184 (0.062 - 0.060 - 0.062)
1 Month	0.206 (0.072 - 0.068 - 0.066)	0.161 (0.056 - 0.053 - 0.052)	0.218 (0.075 - 0.072 - 0.071)

Patient 3	Hair 1 (Mm) Total (Proximal - Intermediate – Distal)	Hair 2 (Mm) Total (Proximal - Intermediate – Distal)	Hair 3 (Mm) Total (Proximal - Intermediate – Distal)
Baseline	0.176 (0.056 - 0.064 - 0.056)	0.152 (0.056 - 0.050 - 0.046)	0.165 (0.058 - 0.056 - 0.051)
1 Month	0.157 (0.051 - 0.056 - 0.050)	0.169 (0.057 - 0.057 - 0.055)	0.197 (0.070 - 0.067 - 0.060)

In the first patient, measurements show a difference of +0.063 mm, which means a 12.90% increase in hair thickness. In the second patient, the difference is +0.037 mm, which means a 6.75% increase in hair thickness. And in the third patient, measurements show a difference of +0.03 mm, which means a 6.08% increase in hair thickness.

Hair Loss Test results are summarized in Table 4.

Table4. *Hair Loss Test.*

Patient	Hair Loss Before Treatment (No. of Hairs)	Hair Loss After Treatment (No. of Hairs)	Difference (%)
1	23	14	39.13
2	19	13	31.57
3	17	5	70.58

The histological study with hematoxylin-eosin showed a slight increase in the epidermis thickness, an increase in the amount of perifollicular collagen fibers and an increase in the amount of existing vessels in the follicle bulb. Using immunohistochemical techniques, [11] the following differences between both groups were observed: an increase in cell mitotic activity with Ki-67 staining, an increase in the amount of perifollicular vascular structures (CD34 staining), an increase in the amount of collagen fibers and fibroblasts in the dermis (vimentin).

DISCUSSION

An increase in the mean of hair thickness, together with reduction of its loss, have been objectified; on the other hand, immunohistological findings are encouraging. Usually, a reduced amount of Ki-67 immunoreactive cells is observed during involution of hair follicles. This denotes a suppression of hair growth and reflects the atrophy of the hair follicle. Cases treated with the Rigenera® system, however, showed an increase in mitotic activity, suggesting higher metabolic activity consistent with the hair growth phase. Likewise, CD34 and Vimentin stains were also quantitatively greater during post treatment, reflecting an increase in perifollicular vascularization, as well as an increase in fibroblastic activity in the adjacent area to the hair bulb.

Methodologically speaking, this small number of cases provides conclusions that are extremely

interesting, but with a very limited scope. The sample size, lack of a control group, and short duration of the observation period determine how to interpret the results obtained.

In conclusion, given the results, using the Rigenera® system seems to be a promising option to treat and slow down the evolution of AGA. However, controlled, randomized, longer clinical trials, with a larger sample, control and placebo groups and quantifiable methods are necessary to irrefutably corroborate these findings.

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CLINICAL AND HISTOLOGICAL EVALUATION OF THE REGENERA® METHOD FOR THE TREATMENT OF ANDROGENETIC ALOPECIA

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ABSTRACT

Androgenetic alopecia has become a more common condition in society, affecting both genders. It is a disorder of multifactorial origin, with therapeutic options both in the rise and under development. Known options include the procedures of regenerative medicine with promising results. This paper assesses clinical and histological changes in patients with AGA after applying an autologous cellular suspension obtained using the Rigenera® system. After applying treatment, an increase in the mean of hair thickness, together with reduction of its loss, have been objectified; the level of satisfaction described by patients is worth noting. Based on the results, the improvement of AGA obtained with the Rigenera system is objective; these results need to be completed with data from future studies after using this promising technique.

KEYWORDS: Alopecia. Regenerative medicine, AGA, Rigenera.

1. INTRODUCTION:

Both male and female androgenetic alopecia (AGA), also known as common baldness, is the most frequent condition in our society. It is estimated to have affected 80% of Caucasian males and approximately 42% of women in the 90s. [1,2] Both genders share its causes, which are mainly genetic inheritance, hormones and aging of the hair follicle.

Hair follicles are complex structures that go through different biological stages: from an active growth stage (anagen phase) and an intermediate remodeling stage (catagen phase), to a quiescent stage (telogen phase). [2] The pathogenesis of androgenetic alopecia is characterized by a shortening of the anagen phase and an increase in the amount of hair follicles that remain in the telogen phase. Since the anagen phase determines hair's length, the new hair in AGA is shorter, gradually miniaturizing hair follicles until they disappear. [3,4] In male pattern AGA, a receding front line is observed, mainly of triangular shape, followed by thinning at the vertex area. [5] The so-called female pattern is characterized by diffuse thinning of the central-parietal region and preservation of hair front line. [1]

AGA is a disorder of multifactorial origin, in which genetics plays an important role. In males, it is an androgen-dependent feature since the terminal follicle becomes susceptible to Dihydrotestosterone (DHT), shortening the anagen phase; whereas in women, the associated hormonal mechanisms are less evident. [2]

Hair density loss needs follow-up and continued treatment. In this sense, therapeutic options —prescribed or natural —promoting hair growth have recently experienced a great rise, and hair regeneration has become one of the main goals of developing therapies. [5]

The purpose of this paper is to objectively assess changes in the scalp, hair bulbs and hair in a number of patients with AGA, after applying an autologous cellular suspension obtained using the Rigenera® system. The treatment involves the administration of intradermal injections in the affected area of one single application of an autologous cellular suspension obtained using the Rigenera® system (Human Brain Wave SRL, Turin, distributed in Spain by the Regenera Activa company) from an autologous skin graft.

2. MATERIALS AND METHODS:

The treatment consists of the mechanical disintegration of a sample of tissue obtained by a skin punch and subsequent filtration (50 microns) to be intradermally administered in the affected area according to technical specifications (Regenera® Protocol, Rigenera® System, Human Brain Wave SRL, Turin).

For this descriptive study, 17 patient volunteers were consecutively and randomly recruited. The defined inclusion criteria were: males and females over 18 years old diagnosed with AGA. Of the 17 patients that were treated with the Rigenera® system, nine were males aged between 24 and 54. According to the scale of Hamilton, they all showed the following male pattern: three had type III, two had type IV, three had type V, and one had type VI. Of the 17 patients treated, eight were women aged between 21 and 58, with female pattern androgenetic alopecia. According to the Ludwig scale, they were classified as follows: two had type III, and six reported hair loss, although without a diagnose of AGA.

Exclusion criteria included: allergy to lidocaine, healing issues, scarring alopecia, chronic drug treatment, oncologic processes and having performed any hair loss treatment topical, oral or injectable between 3 months prior and 3 months after the date of applying treatment except taking vitamin supplements and applying topical lotions or shampoos. The application was made on the scalp using mesotherapy. In males, the treatment was applied in areas 2, 3, 4, 5 and 6, while in females, it was applied in all ten areas (fig. 1).

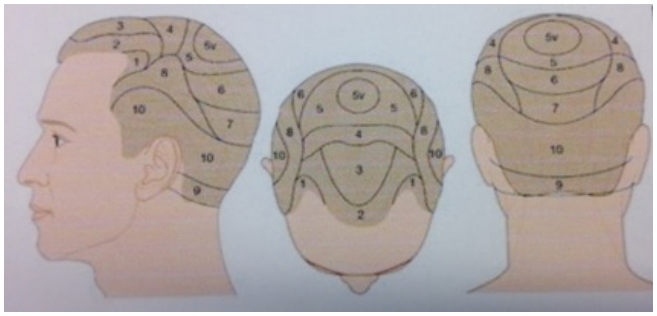

Figure 1: Application areas

Control visits were conducted prior to treatment and 30 days after its application. Eight pictures per session were taken for analysis in a photographic studio. These were assessed by a doctor specialized in hair medicine, who was not part of the study. The variables assessed were:

1. Evidence of improvement: comparison of pictures taken before treatment with those taken 30 days later (Table 1).

2. Assessment of improvement according to scales of Hamilton and Ludwig.

Patients' level of satisfaction with the treatment was recorded as well, along with their perception of changes in hair thickness, density, and loss (table 1). Lastly, immediately after each session, patients recorded the level of pain during application.

3. RESULTS:

The analysis of the photographic evaluation (fig. 2) showed an improvement of hair density in four patients, that is 23.53%. Thirteen patients (76.47%) didn't notice any change, and none of them noticed worsening of hair density.

As for the subjective assessment of satisfaction, of the 17 patients treated, one patient was very satisfied (5.88%), five patients were quite satisfied (29.41%), eight patients were satisfied (47.05%), one patient was somewhat satisfied (5.88%) and two were unsatisfied (11.76%).

Concerning patients' perception of change in hair thickness, 12 patients observed an increase in thickness (70.58%) and five did not observe any change (29.41%).

Concerning patients' perception of change in hair loss, no increase was observed: nine did not notice any change (52.94%) and eight observed a decrease in hair

loss (47.05%).

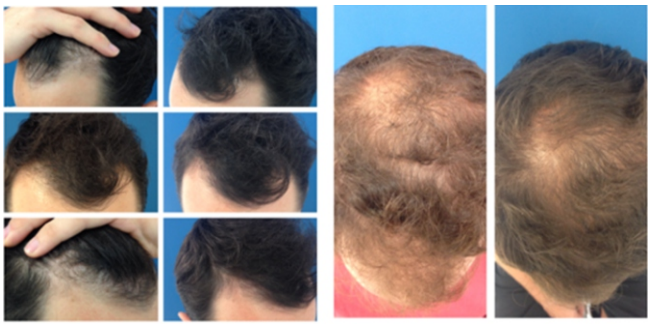

Figure 2: pre and post pictures. Left: case 1 (Day 0 top - Day 30 bottom). Right: case 2 (Day 0 left - Day 30 – right).

Table 1: Patient perception scale.

	Thickness	%	Fall	%	Density	%
Increase	12	70.58	0	0	4	23.53
No change	5	29.41	9	52.94	13	76.47
Decrease	0	0	8	47.05	0	0

For pain perception during treatment, one patient indicated a level 0 (5.88%), two patients indicated a level 2 (11.76%), seven patients indicated a level 3 (41.17%), five patients indicated a level 5 (29.41%), one patient indicated a level 6 (5.88%) and one patient indicated a level 10 (5.88%).

4. DISCUSSION:

This study evaluated changes occurring after application of the Rigenera® system in patients with AGA, or that currently suffered from significant hair loss. Said changes have been assessed in the scalp and hair bulbs using the histological study of skin biopsies and hair units; changes in hair density have been assessed using macroscopic and microscopic photographic studies; changes in hair thickness using micrometer measurements, and changes in hair loss using a Hair Loss Test.

An increase in the mean of hair thickness after application of one single therapeutic session has been objectified, which, together with a decrease in hair loss (according to the Hair Loss Test) in the same patients, suggest a certain improvement of alopecia in treated cases. It is worth noting that the levels of satisfaction most described by patients are Satisfied, Quite Satisfied or Very Satisfied with the treatment, considering its cost.

The sample size, lack of a control group, and extension of the observation period determine how to interpret the results obtained and their validity. Due to the complexity of the hair growth cycle, in order to properly evaluate the improvement in hair density, future studies should assess the trichogram as proof of analysis and perform assessments three months after treatment. Another key point is to standardize the conditions under which pictures are taken.

In general, patients' subjective assessment of the results was positive, describing an improvement mainly in thickness and hair loss, being a well-tolerated treatment concerning pain, and with no side effects. However, controlled, randomized, longer clinical trials, with a larger sample, control and placebo groups and quantifiable methods are necessary.

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Video Article

Tissue Characterization after a New Disaggregation Method for Skin Micro-Grafts Generation

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URL: http://www.jove.com/video/53579
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Keywords: Medicine, Issue 109, Micro-grafts, Rigenera protocol, skin, cell viability, stem cells, bio-complexes

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Abstract

Several new methods have been developed in the field of biotechnology to obtain autologous cellular suspensions during surgery, in order to provide one step treatments for acute and chronic skin lesions. Moreover, the management of chronic but also acute wounds resulting from trauma, diabetes, infections and other causes, remains challenging. In this study we describe a new method to create autologous micro-grafts from cutaneous tissue of a single patient and their clinical application. Moreover, *in vitro* biological characterization of cutaneous tissue derived from skin, de-epidermized dermis (Ded) and dermis of multi-organ and/or multi-tissue donors was also performed. All tissues were disaggregated by this new protocol, allowing us to obtain viable micro-grafts. In particular, we reported that this innovative protocol is able to create bio-complexes composed by autologous micro-grafts and collagen sponges ready to be applied on skin lesions. The clinical application of autologous bio-complexes on a leg lesion was also reported, showing an improvement of both re-epitalization process and softness of the lesion. Additionally, our *in vitro* model showed that cell viability after mechanical disaggregation with this system is maintained over time for up to seven (7) days of culture. We also observed, by flow cytometry analysis, that the pool of cells obtained from disaggregation is composed of several cell types, including mesenchymal stem cells, that exert a key role in the processes of tissue regeneration and repair, for their high regenerative potential. Finally, we demonstrated *in vitro* that this procedure maintains the sterility of micro-grafts when cultured in Agar dishes. In summary, we conclude that this new regenerative approach can be a promising tool for clinicians to obtain in one step viable, sterile and ready to use micro-grafts that can be applied alone or in combination with most common biological scaffolds.

Video Link

The video component of this article can be found at http://www.jove.com/video/53579/

Introduction

In the last years, several new methods have been developed in the field of biotechnology to obtain autologous cellular suspensions during surgery in order to provide one-step treatments for acute and chronic skin lesions. Moreover, the management of acute but mainly chronic wounds resulting from trauma, diabetes, infections, and other causes, remains challenging. There is mounting evidence that chronic wounds have become a serious global health issue, causing an enormous financial burden on healthcare systems worldwide[1].

To increase the rate of success in the treatment of skin lesions, the absence of extensive manipulation (including cellular enhancement) and the maintenance of sterile conditions are essential, in order to create a cellular suspension that can be immediately applied on the damaged area of the patients, thereby avoiding a longer processing in cleanrooms such as Cell Factories. Starting from small skin biopsies, grinding, centrifugation and other separation methods (*e.g.,* enzymatic or mechanical), are frequently used to obtain a cellular suspension, which can be cultured in a growth medium. All of these methods generally require a long time of execution, stressing the cell structures, and leading to a reduction of cell viability. Another significant aspect is to obtain an autologous cellular suspension ready to be used by clinicians, for example, to repair damaged areas. Furthermore, it is well established that autologous tissue grafts survive the transfer procedures to eventually survive in the recipient site by the principles of induction and conduction [2, 3]. The ideal graft tissue should be readily available and have low antigenicity and donor site morbidity [4].

On the basis of these evidences, the first aim of this study was to create autologous bio-complexes suitable for clinical application in the tissue repair. For this purpose, we describe a new method to obtain autologous micro-grafts starting from cutaneous tissues which were disaggregated by this protocol. A case presentation is also herein described as a clinical application of autologous micro-grafts obtained by this protocol in combination with collagen sponges. This approach has already been reported to be efficient in the mechanical disaggregation of human tissues[5] and it has been used clinically for grafts and regeneration of dermal tissues [6,7] as well as for regenerative therapies of connective tissues in oral-maxillofacial surgery [8-10].

In addition, the second aim of this study was the biological characterization of the cutaneous tissues after their disaggregation by this protocol. To this purpose, different homologous samples of cutaneous tissue derived from the trunk area of different multi-organ and/or multi-tissue donors were processed following National Rules on harvesting, processing and distributing tissues for transplantation (CNT 2013) at Emilia Romagna Regional Skin Bank.

CASE PRESENTATION:

A 35-year-old female patient showing a complex trauma due to car accident was admitted to the Intensive Care Unit of Ancona Hospital. The patient showed an infection on the leg due to an open wound and a compound fracture stabilized with external fixation. Two radical debridement were performed and when the wound became clean after negative pressure therapy (V.A.C. therapy) and the periosteum appeared healthy, we applied the protocol after two months from recovery. After disaggregation with this system, the micro-grafts obtained were used to create bio-complexes with a collagen sponge which were subsequently implanted in order to investigate their efficacy on the lesion repair.

Protocol

Ethics statement: since the clinical application of the protocol requires the use of cutaneous autologous tissue of the patient, its characterization *in vitro* was performed before clinical use on homologous cutaneous tissue at Emilia Romagna Regional Skin Bank following the guidelines of National Rules on harvesting, processing and distributing tissues for transplantation (CNT 2013).

1. Bio-complex Building for Clinical Application

NOTE: This protocol is clinically based on the use of Rigeneracons (tissue disruptor) and the Rigenera Machine (tissue disruptor system) (**Figure 1A**). The tissue disruptor is a biological medical disruptor of human tissues able to disrupt small pieces of tissues using a grid provided by hexagonal blades and filtering cells and components of extracellular matrix with a cut-off of about 50 microns.

1. Collect skinsamples of the patient through a biopsy punch (**Figure 1B**) and disaggregate adding 1 ml of saline solution for each piece to obtain autologous micro-grafts [6,7,9] (or see step 2.1).
2. Place 1 ml of micro-grafts on collagen sponges (**Figure 1C**) toform bio-complexes to use for clinical application.
3. Culture another 1 ml of micro-grafts on collagen sponges in the presence of 6 ml of DMEM medium supplemented with 10% Fetal Bovine Serum at 37 °C in a 5% CO_2 humidified atmosphere.
4. Following 3 days of culture, fix the bio-complexes with 0.3% Paraformaldehyde for 10 min at RT. Pour the paraffin with a specific dispenser directly onto the sample. Obtain slices with a microtome with a thickness of 5 µm and put directly in a glass-slide.
5. Immerse paraffin slices of 5 µm in a glass for histological analyses, containing 15 - 20 ml Xylene (commercially available - a mix of m-xylene (40 - 65%), p-xylene (20%), o-xylene (20%) and ethyl benzene (6 - 20%) and traces of toluene, trimethyl benzene, phenol, thiophene, pyridine and hydrogen sulfide) for 3 min each.
6. Immerse the slices in decreasing grades (100% to 70%) of ethanol (100% ethanol for 1hr, 95% ethanol for 1 hr, 80 % ethanol for 1hr, 70% ethanol for 1 hr) and then deionized water for de-paraffinizing and rehydrating the sections.
7. Stain the sections with 1 - 2 ml of 1g/L Ematoxylin for 1 - 2 min and subsequently rinse in water to remove any Ematoxylin surplus.
8. Stain the sections for 4 - 5 min with Eosin Y alcoholic solution at 1% of concentration mixed with ethanol 70% and diluted in water.
9. Use 1 - 2 ml of Eosin Y for each slide section and rinse under running tap water.
10. Immerse sections in increasing grades of ethanol (see step 1.6) and finally, after a passage in Xylene for 1h, coverslip with a based-mounting mediumand observe under a light microscope at 100X magnification (**Figure 1D**).

2. Collection, Disaggregation and *In Vitro* Analysis of the Tissues

1. Using a dermatome, take independent skin tissue, papillary de-epidermized dermis (Ded) or reticular dermis (Dermis) respectively of 0.6 mm, 1 mm or 2 mm in thickness from the trunk area of 4 different multi-organ and/or multi-tissue donors in a range from 40 to 55 years, following National Rules on harvesting, processing and distributing tissues for transplantation (CNT 2013).
    1. Gently rinse all samples in 0.9% NaCl solution putting them in a dish on an orbital shaker for 5 min.
    2. Using the 5-mm biopsy punch, create samples which are uniform in diameter from the skin tissue, Ded and Dermis and weigh all tissue specimens before the disaggregation.
    3. Insert eight, three or four uniform samples of skin tissue, Ded or Dermis respectively, in the tissue disruptor, adding 1.5 ml of saline solution for the disaggregation.
    4. Perform different times of disaggregation for all tissue samples as indicated in **Table 1**.
    5. Use a correspondent number of punch biopsies derived from intact tissue samples as controls.
    6. After mechanical disaggregation, aspirate the saline solution containing micro-grafts and place separately each sample in a single well of a 12-well plate. Perform the same protocol for intact control punch biopsies.
    7. Add 1ml of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics to each sample.
    8. Evaluate the cell viability immediately. To each well containing a micro-graft (obtained by the simultaneous disaggregation of eight, three or four uniform samples of skin tissue, Ded or Dermis respectively) add 1ml of medium containing 0.5 mg/ml of MTT (3-[4,5-

dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) solution and incubate for 3 hr at 37 °C in an atmosphere of 5% CO_2/air. Perform the same protocol for intact control punch biopsies.
9. After incubation, remove all medium containing MTT and add to each sample 1 ml dimethyl sulfoxide (DMSO) for 10 min.
10. Transfer each sample and DMSO in a cuvette and read at optical density (OD) at 570 nm using a spectrophotometer. Calculate cell viability as the ratio of absorbance at 570 nm and the weight in grams (gr) of tissue used before disaggregation. Perform the same protocol for intact control punch biopsies.

2. After mechanical disaggregation, aspirate the saline solution containing micro-graft derived from skin tissue, Ded and Dermis samples of a single donor.
    1. Place separately each sample in a single well of a 12-well plate or in a culture flask for cell viability test and morphological analysis respectively.
    2. Culture micro-grafts adding 1 ml (12-well plate) or 5 ml (culture flask) of RPMI 1640 medium supplemented with 10% FBS and antibiotics at 37 °C in an atmosphere of 5% CO_2/air for 24 hr or 7 days.
    3. Evaluate the cell viability after 24 hr or 7 days (repeat steps 2.1.8-2.1.10).
    4. Perform morphological analysis evaluating the presence of cell suspension by light microscopy after 24 hr and 7 days of culture in flask.
    5. Analyze the samples of Dermis for positivity to the mesenchymal and hematopoietic cell markers, including CD146, CD34 and CD45 antigens by FACS analysis [6].
    6. Under laminar flow hood seed each micro-graft (obtained by the simultaneous disaggregation of eight, three or four uniform samples of skin tissue, Ded or Dermis respectively) and a correspondent small fragment of each tissue sample not totally disaggregated (>50 micron in size after disaggregation process) on Columbia agar plate containing 5% sheep blood broth 100 µl.
    7. Incubate the plate at 37 °C for three days and perform microbiological analysis on Columbia agar plate in order to assess the sterility[11].

Representative Results

In this preliminary study, the first aim was to investigate the ability of human autologous micro-grafts combined with a biological support, such as collagen, to produce bio-complexes ready to use. These bio-complexes were implanted in a patient with a leg lesion caused by a car accident **(Figure 2A**) and a complete re-epithelialization associated with tissue repair after 30 days (**Figure 2B)** was observed. Moreover, the clinical follow-up showed a good texture and softness of the damaged area after 5 months (**Figure 2C).** In parallel to clinical application, *in vitro* studies were also performed to evaluate the cell viability of different cutaneous tissues such as skin tissue, Ded and Dermis before and after disaggregation by this system. In particular, taking into account the different thickness of each type of tissue, the threshold of eight, four and three biopsies that can be processed in a single step for skin tissue, Ded and Dermis, respectively, was established. The established number of biopsies were then disaggregated at four different times as indicated in the **Table 1**, according to their biological characteristics in order to identify the optimal condition of disaggregation to maintain a good cell viability.

Although the tissue processing itself inevitably induced an impairment of cell viability compared to intact tissue, the mechanical disaggregation performed with this system seems to maintain a mean value of cell viability of 30% in all samples of skin, Ded and dermis evaluated at different times **(Figure 3A),** with respect to intact tissue (**Figure 3B**) (mean of 92 OD/gr for intact skin tissue and 29 OD/gr after disaggregation; from 22 OD/gr to 7 OD/gr for intact Ded with respect to disaggregation and finally from 16 OD/gr to 2.7 OD/gr for intact dermis with respect to disaggregation). Thus, these preliminary results show that this system is able to maintain appreciable levels of cell viability after immediate disaggregation. The effect of disaggregation on cell viability was also investigated on tissue samples cultured for 24 hr or 7 days and variable results were observed. In particular, no substantial variation of cell viability in skin tissue samples was observed independently by time of homogenization and culture compared to starting time (T_0) **(Figure 4A**). On the other hand, a reduced viability of Ded samples after 24 hr of culture compared to starting time (T_0) was observed but surprisingly the cell viability was restored after 7 days of culture (**Figure 4B**). Similar results were also observed for samples of Dermis (**Figure 4C**). Each sample was evaluated in duplicate and values reported in the **Table 2**.

On the basis of these results, we identified the suitable time of disaggregation to maintain cell viability for three types of cutaneous tissue as reported in **Table 3.** In addition to cell viability, the morphological aspect of cultured cellular suspension was also evaluated, identifying single cells after 24 hr from tissue disaggregation, while after 7 days there residues of fibers in both skin tissue and Ded/Dermis samples were observed (**Figure 5 A-B**). In addition, a cell characterization by flow cytometry analysis was performed in a sample of dermis after its homogenization: a heterogeneous pool of cells composed by several cellular types, including endothelial cells and mesenchymal stem cells was identified (**Figure 6**). Furthermore, the absence of bacterial growth was identified in all disaggregated samples opportunely seeded on Agar dishes, evidencing the sterility of experimental procedure (**Figure 7**).

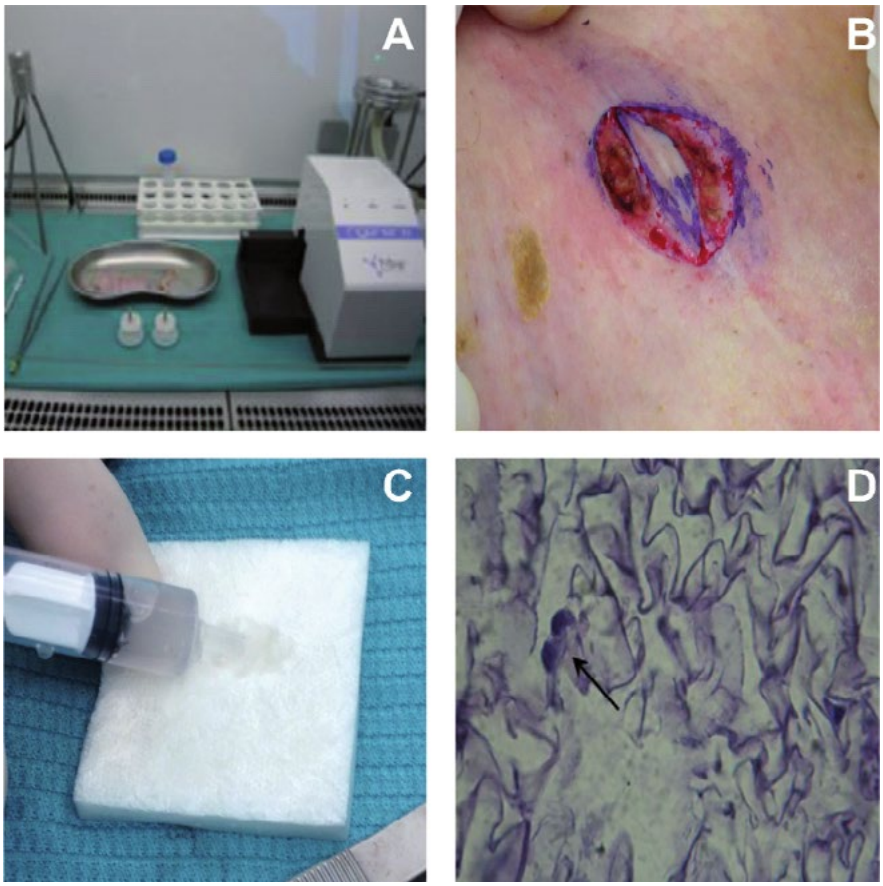


Figure 1. Bio-complexes Building Tissue Disrupting System (A) and Collection of a Small Piece of Derma from the Lesion Area of the Patient that was Subsequently Disaggregated with Tissue Disruptors. The bio-complexes were obtained combining the micro-grafts on collagen sponges to obtain human patches ready to use for lesion repair **(C)**. **(D)** Hematoxylin & eosin (H&E) staining of a representative bio-complex cultured for three days in DMEM medium and observed to microscopy Please click here to view a larger version of this figure.

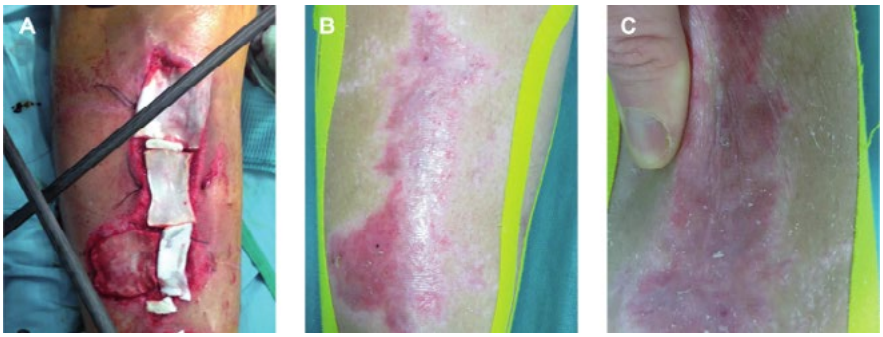


Figure 2. Bio-complexes Application on Leg Lesion The bio-complexes composed by collagen and micro-grafts obtained from the patient were applied on the leg lesion **(A)** and the wound was evaluated after 30 days **(B)** and 5 months **(C)**. Please click here to view a larger version of this figure.

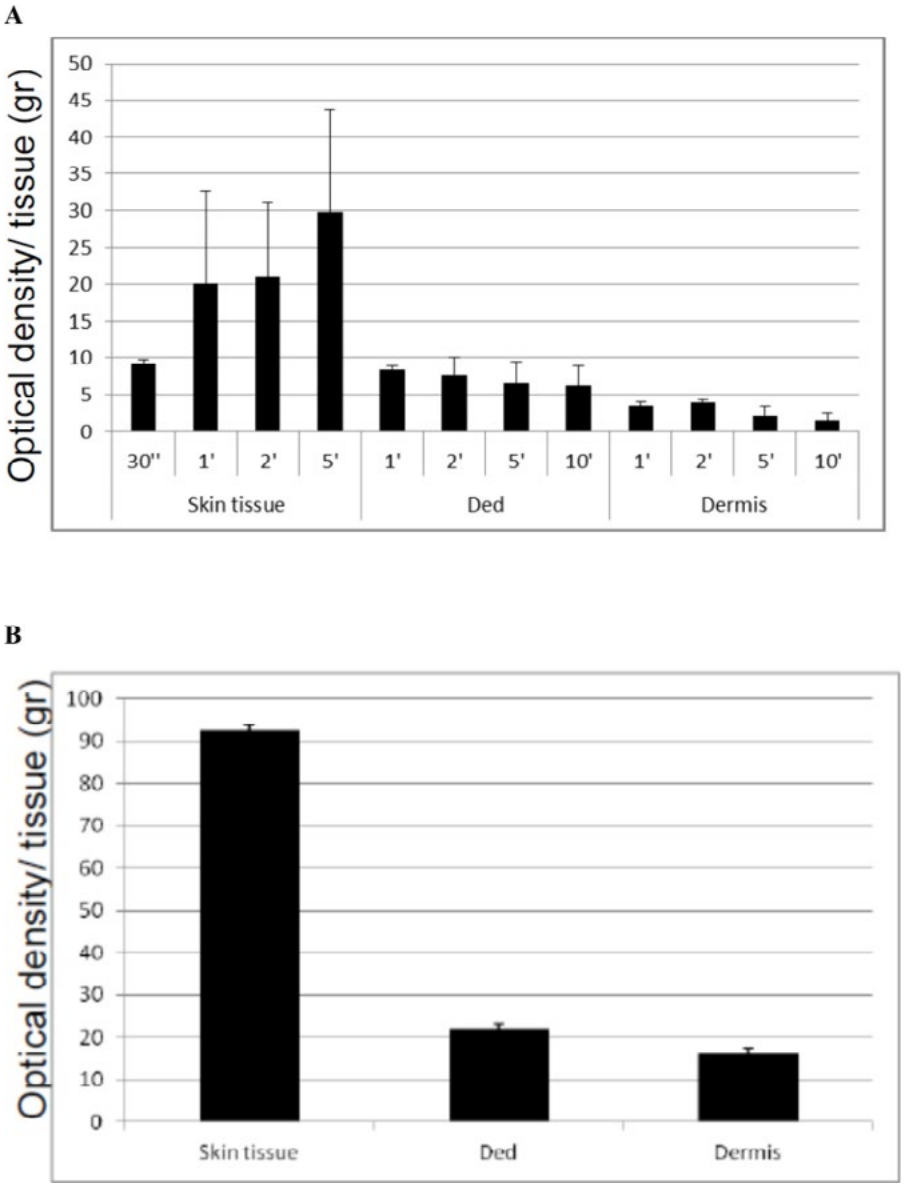


Figure 3. Cell Viability Immediately after Disaggregation at Different Times of Three Type of Cutaneous Tissues (A) with respect to Intact Cutaneous Tissues (B). The Skin, Ded and Dermis tissues derived from four different donors were disaggregated with tissue disruptors as indicate in the appropriate section in the text. Cell viability was assessed by MTT test performed in duplicate for each tissue sample. The graph is representative of four different experiments performed in duplicate for each sample. The results are expressed as a ratio between Optical Density (OD) and grams (gr) of tissue; the standard deviation was calculated on the ratio between Optical Density (OD) and grams (gr) of tissue. Please click here to view a larger version of this figure.

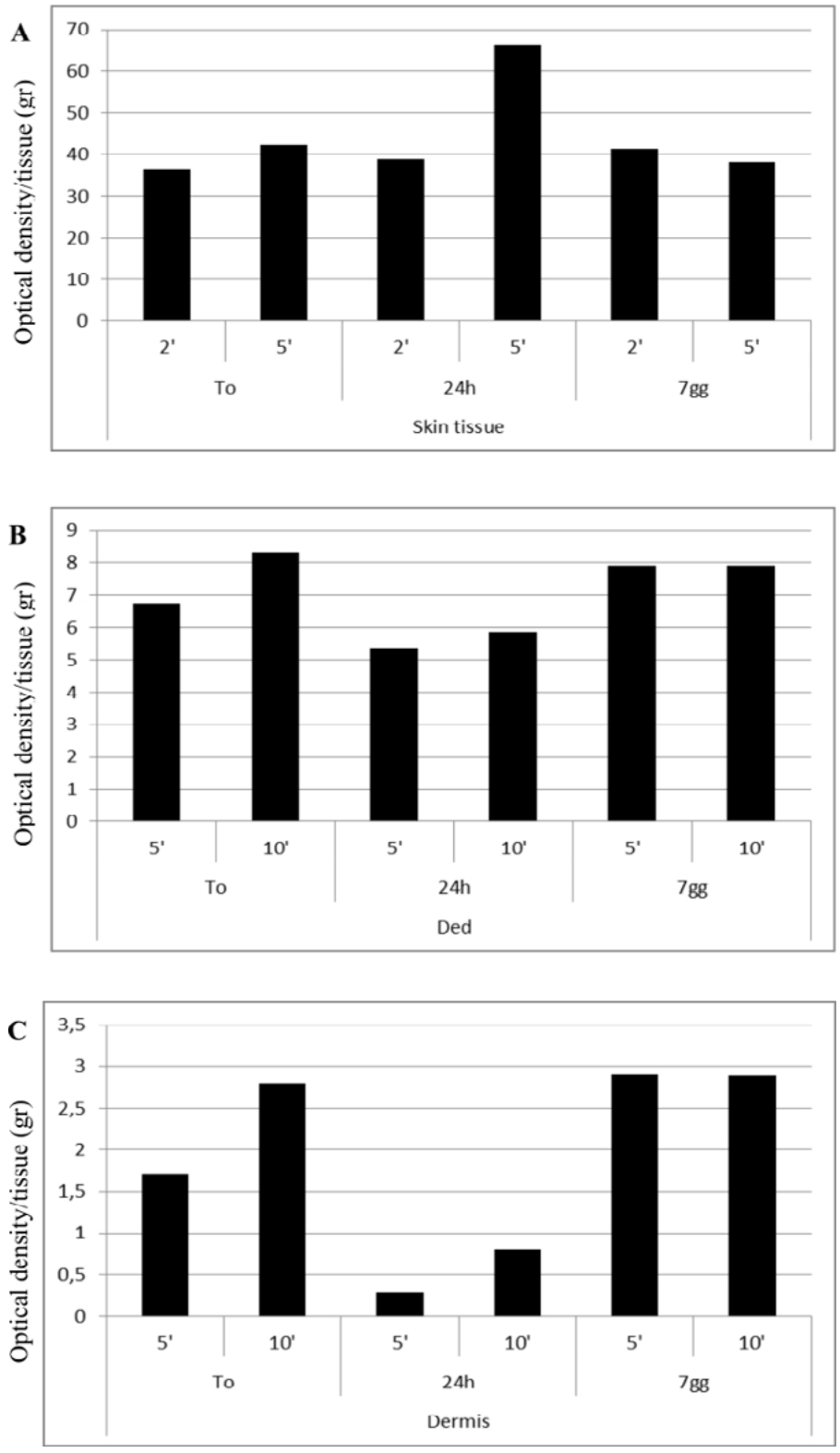


Figure 4. Cell Viability Levels of a Disaggregated Sample of Skin Tissue (A), Ded (B) and Dermis (C) to Starting Time (T_0) and after Subculture for 24 hr and 7 Days. The tissues were homogenized with tissue disruptors as indicate in the appropriate section in the text. The disaggregated samples were subsequently cultured for 24 hr and 7 days after the disaggregation and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C in an atmosphere of 5% CO2/air. Cell viability was assessed by MTT as previously described. The graph is representative of an experiment performed in duplicate for each sample of skin tissue, Ded and Dermis derived from a single donor. The results are expressed as ratio between Optical Density (OD) and grams (gr) of tissue. Please click here to view a larger version of this figure.

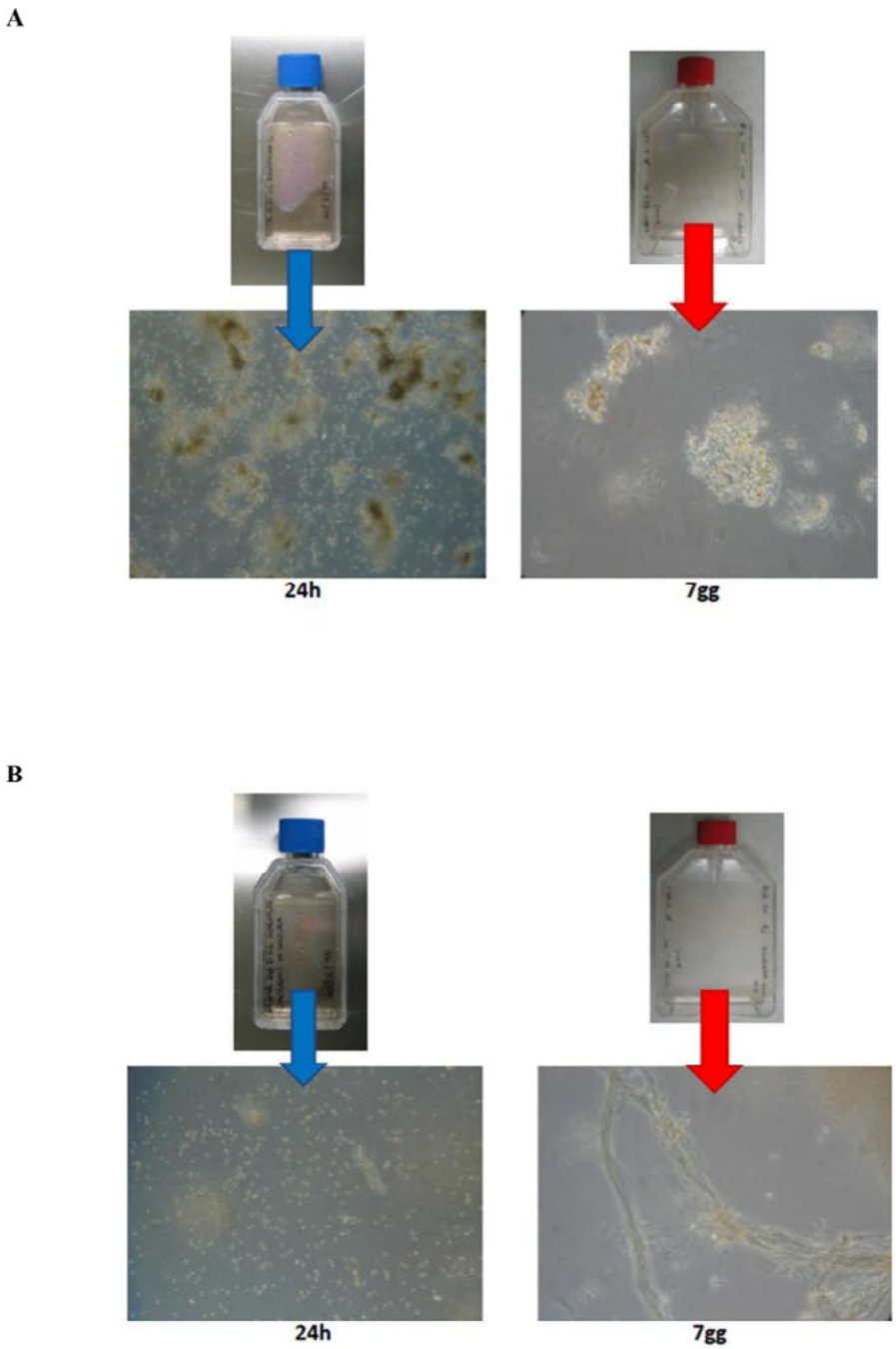
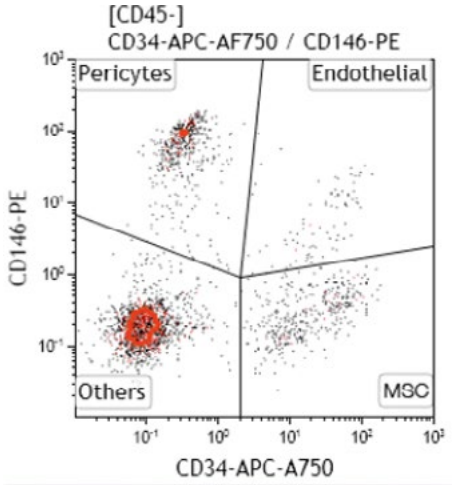


Figure 5. Morphological Analysis of Disaggregated Skin Tissue (A) and Ded/Dermis (B) after 24 hr and 7 Days of Culture. Morphological analysis was performed using light microscopy after 24 hr and 7 days of culture in the presence of RPMI medium on skin tissue and Ded/dermis tissues. Please click here to view a larger version of this figure.



Gate	Number	%Total	%Gated
All	2,362	9.96	100.00
MSC	382	1.61	16.17
Endothelial	83	0.35	3.51
Others	1,412	5.95	59.78
Pericytes	485	2.04	20.53

Figure 6. Cell Characterization by Flow Cytometry Analysis. After mechanical disaggregation, micro-grafts obtained by dermis were put in culture and after 7 days were analyzed for positivity to mesenchymal and hematopoietic cell marker, including CD146, CD34 and CD45 antigens. Please click here to view a larger version of this figure.

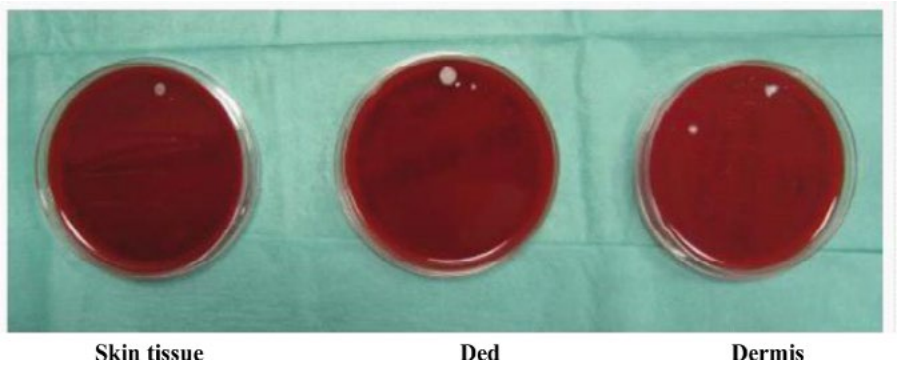


Figure 7. Microbiological Analysis of Disaggregated Skin Tissue, Ded and Dermis Samples. Small fragments of disaggregated tissue samples were seeded on Columbia agar added of 5% sheep blood (BioMerieux Company) under laminar flow hood and incubated at 37 °C for three days to perform the microbiological analysis and assess the sterility of procedure. Please click here to view a larger version of this figure.

TISSUE SAMPLES	DISAGGREGATION TIMES (sec/min)
Skin	30 sec
	1 min
	2 min
	5 min
Ded	1 min
	2 min
	5 min
	10 min
Dermis	1 min
	2 min
	5 min
	10 min

Table 1. Schematic Representation of the Four Different Times used for Disaggregation of Skin, Ded and Dermis. Eight, four and three punch biopsies of Skin, Ded and Dermis respectively were disaggregated at four different times, according to their biological characteristics.

Skin tissue					
To		**24 hr**		**7 days**	
2'	**5'**	**2'**	**5'**	**2'**	**5'**
37.1	41.07143	41.07143	65.78571	41.14286	37.42857
35.8	43.82143	36.60714	66.5	41.46429	38.67857
Ded					
T_0		**24 hr**		**7 days**	
5'	**10'**	**5'**	**10'**	**5'**	**10'**
6,649485	8.237113	5.463918	5.731959	7.835052	7.85567
6,845361	8,360825	5.257732	5.989691	8	7.938144
Dermis					
To		**24 hr**		**7 days**	
10'	**5'**	**10'**	**5'**	**10'**	
1.690909	2.77193	0.293898	0.786704	2.880952	2.869048
1.736364	2.830409	0.306351	0.814404	2.940476	2.928571

Table 2. Range of Values Expressed in OD/gr for One Biological Replicate. Values of cell viability from skin tissue, Ded and dermis evaluated to starting time and after mechanical disaggregation to indicated times. The results are representative of one experiment performed in duplicate and expressed as ratio between Optical Density (OD) and grams (gr) of tissue.

Specimen	**Disaggregation Time**
Skin tissue	5 min
Ded	1 min
Derma	2 min

Table 3. Schematic Representation of the Best Time of Disaggregation to Maintain a Good Cell Viability in Different Tissue Samples. Skin, Ded and Dermis tissue samples show the optimal cell viability after 5, 1 and 2 min of disaggregation, respectively.

Discussion

This preliminary study showed that micro-grafts obtained by this protocol can be combined with collagen sponges, as already reported in other clinical applications, to optimize the efficacy of micro-grafts implants[9, 10]. In particular, this study reported the capacity of bio-complexes, constituted by micro-grafts and collagen sponges, to adjuvant the wound healing of a leg lesion after 30 days from clinical application. Furthermore, *in vitro* results provide evidence about the effectiveness of this protocol to disaggregate three different cutaneous tissues, maintaining an appreciable cell viability both immediately after the mechanical disaggregation and also after 24 hr and 7 days of culture.

The maintenance of cell viability after this kind of homogenization allows to obtain in only one step sterile autologous micro-grafts, avoiding extensive manipulation. This evidence is in agreement with other recent papers in which the efficacy of this protocol *in vitro* was tested also in other type of tissues, including periosteum, biopsy of cardiac atria and lateral rectus muscle of eyeball[5]. In particular, we observed in Ded and dermis samples an increased cell viability after 7 days of culture, probably due to the use of a medium supplemented with 10% fetal bovine serum.

In addition to the maintenance of cell viability, this system is safe and easy to use and in a few minutes is able to mechanically disaggregate different types of tissues preventing the disruption of cell structures, with respect to traditional methods of cell isolation, such as enzymatic digestion that require longer time of execution. Furthermore, this system is able to select cells with a cut-off of 50 microns and this aspect is very important in consideration of the success of injectable micro-grafts, because this range includes progenitor cells able to differentiate in many cells types and then ameliorate the lesion repair. This protocol allows us not only to obtain viable but also autologous micro-grafts, given that the donor subject is also the acceptor of these micro-grafts. The capacity of this system to obtain autologous micro-grafts was especially reported in the field of dentistry where it has been shown that transplantation of autologous Dental Pulp Stem Cells (DPSCs) in a non- contained infrabony defect contributed to periodontal repair and regeneration of atrophic maxilla[5,6]. The ability of these micro-grafts to improve the wound healing process was also previously reported in the management of complex wounds after surgical interventions or complications[4].

Another advantage on the use of autologous and ready to use micro-grafts is certainly the keeping of sterile conditions in view of their subsequent implant on the skin lesions.

In conclusion, the protocol described in this paper showed the capacity to create human micro-graft tissue ready to use in clinical intervention to improve healing of acute/chronic skin wounds, such as those indicated in this study. *In vitro* results on the possibility to create viable micro-grafts have also been reported and this parameter is certainly significant to increase the percentage of success in the tissue repair. Taken together, the data showed that this new system could be considered a promising tool for clinicians who are in need of a rapid and safe instrument in the management of skin wounds.

At the moment, there are not particular modifications or limitations for the protocol in this specific application, given that the skin represents a tissue easy to use and in other studies we reported the efficacy of the same protocol in different skin lesions [6,7]. We hope in the future to use this system on a large number of subjects to validate its clinical use in other clinical areas, (for example the orthopedics for bone regeneration or in vascular medicine for the ulcers treatment).

Disclosures

The author Antonio Graziano is the Scientific Director of Human Brain Wave s.r.l. that produces and markets the Rigenera system. The author Letizia Trovato is a collaborator of Scientific Division of Human Brain Wave s.r.l.

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Stem Cell Research & Therapy

LETTER Open Access

Exosomes derived from human adipose tissue-derived mesenchymal stem cells alleviate atopic dermatitis

Byong Seung Cho, Jin Ock Kim, Dae Hyun Ha and Yong Weon Yi*

Abstract

Exosomes are nano-sized vesicles (30–200 nm) constantly released by almost all cells. The ability of exosomes to travel between cells and deliver their cargo, which includes lipids, proteins, and nucleic acids, makes them an appealing cell-free therapy option to treat multiple diseases. Here, we investigated for the first time whether human adipose tissue-derived mesenchymal stem cell-derived exosomes (ASC-exosomes) can ameliorate atopic dermatitis (AD) in an in vivo mouse model. When injected either intravenously (IV) or subcutaneously (SC) into NC/Nga mice treated with house dust mite antigens, ASC-exosomes were found to reduce pathological symptoms such as clinical score, the levels of serum IgE, the number of eosinophils in blood, and the infiltration of mast cells, CD86+, and CD206+ cells in skin lesions. ASC-exosomes also significantly reduced mRNA expression of various inflammatory cytokines such as interleukin (IL)-4, IL-23, IL-31, and tumor necrosis factor-α (TNF-α) in AD skin lesions of Nc/Nga mice. Taken together, these results suggest that ASC-exosomes can be a novel promising cell-free therapeutic modality for AD treatment.

Keywords: Exosome, Adipose tissue-derived mesenchymal stem cells, Atopic dermatitis, Inflammation

Introduction

Since current treatment options for atopic dermatitis (AD) are limited and have potentially harmful side effects, there are unmet needs to develop novel therapies that are safe and efficacious [1]. Several biologics targeting pro-inflammatory cytokines are currently under development and dupilumab, a dual inhibitor of IL-4 and IL-13, was recently approved by the US FDA for treating adults with moderate to severe AD [1]. Although long-term follow-up study is needed to determine late side effects of dupilumab [1], its efficacy indicates that multiple targeting is a plausible way to treat AD [2].

Several studies have demonstrated that the allergic progress in AD could be suppressed by mesenchymal stem cells (MSCs) derived from human umbilical cord blood (UCB-MSC), bone marrow (BM-MSC), or adipose tissue (ASC) by modulating multiple targets [3]. However, therapeutic use of MSCs has several drawbacks, such as poor engraftment efficiency, potential tumor formation, unwanted immune responses, non-specific differentiation, short half-life, and the difficulty of quality control before administration [4].

Exosomes are nanovesicles (30–200 nm) released by almost all cells and found in all body fluids [4]. Exosomes deliver their cargo (proteins, lipids, and nucleic acids) from originating cells to recipient cells. Growing evidence suggests that exosomes derived from stem cells could be a promising alternative to cell-based therapy because exosomes would avoid most of the problems associated with cell-based therapy while recapitulating the therapeutic efficacy of stem cells [4]. For example, exosomes have no risk of tumor formation as they cannot replicate. They also can be sterilized by filtration and have a longer shelf-life than cells themselves. Being much smaller than stem cells, exosomes easily circulate through the body and reach sites of injury. In addition, long-term repetitive administration of exosomes does not elicit toxicity [5]. Here, we for the first time investigated the therapeutic effect of exosomes derived from human ASC (ASC-exosomes) on AD in a mouse model.

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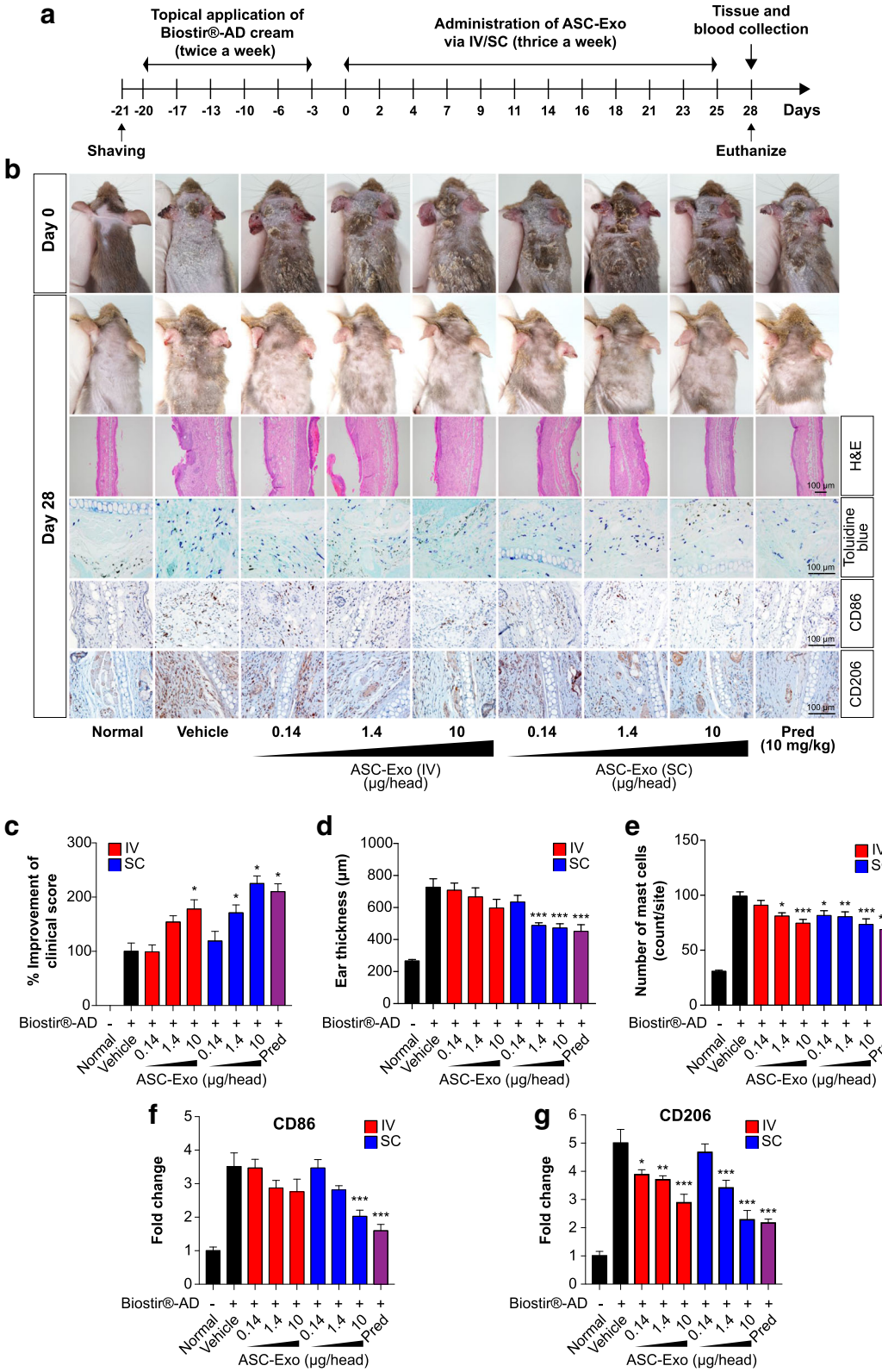

Fig. 1 (See legend on next page.)

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Fig. 1 Therapeutic effects of ASC-exosomes on AD-like lesions in NC/Nga mice. **a** The study protocol. **b** Representative skin manifestations in NC/Nga mice at days 0 and 28, H&E staining results, toluidine blue staining results, and immunohistochemical staining of CD86+ or CD206+ cells of ear skin samples from AD mice. Enlarged images of toluidine blue staining for mast cells and immunostaining for CD86 and CD206 are shown in Additional file 1: Figures S4–S6, respectively. Indicated amounts of ASC-exosomes (micrograms/head) were administered either by IV or SC thrice a week for 4 weeks. **c** Relative percentage improvement of clinical skin severity scores compared to vehicle group. Percentage improvement was calculated as described in Additional file 1: Materials and methods. **d** Improvement of ear thickness as measured in H&E-stained tissue sections in **b**. **e** The number of mast cells in the skin lesions determined in toluidine blue-stained tissue sections in **b**. Quantitative analysis of CD86+ (**f**) and CD206+ (**g**) cells as determined in tissue sections in **b**. Results are presented as mean ± standard error of the mean; $n = 10$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs vehicle control group. *IV* intravenous administration, *SC* subcutaneous administration, *Pred* prednisolone

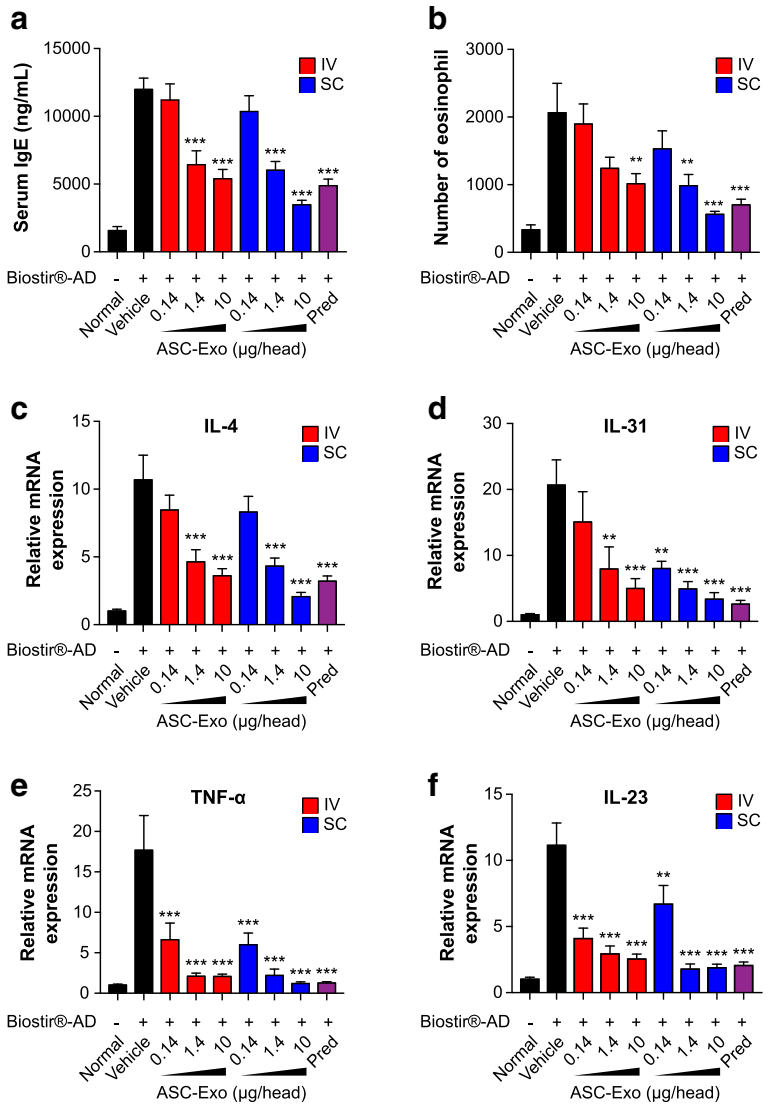

Fig. 2 The effect of ASC-exosomes on the level of serum IgE, the number of eosinophils, and the expression of pro-inflammatory cytokines in NC/Nga mice. **a** The level of serum IgE was detected by ELISA. **b** The number of eosinophils in blood was determined by differential cell counting. **c**–**f** Total RNA was isolated and the mRNA levels of IL-4 (**c**), IL-31 (**d**), TNF-α (**e**), and IL-23 (**f**) were detected by quantitative real-time PCR and normalized by GAPDH mRNA expression. Results are presented as mean ± standard error of the mean; $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs vehicle group. *IV* intravenous administration, *SC* subcutaneous administration, *Pred* prednisolone

Results and discussion

ASC-exosomes were isolated by a sequential filtration method from serum-free conditioned media (Additional file 1: Materials and methods) of ASCs and characterized according to the recommendation of the International Society for Extracellular Vesicles (ISEV) [6]. Transmission electron microscopy analysis and nanoparticle tracking analysis revealed the size distribution and number of ASC-exosomes (Additional file 1: Figure S1A, B). The characteristics of ASC-exosomes were also validated by western blotting with antibodies against surface markers (CD9, CD63, and CD81) and an internal marker (TSG101) (Additional file 1: Figure S1C). CD63 and CD81 were further analyzed by flow cytometry (Additional file 1: Figure S1D). We also used a cell-based assay as an in vitro potency assay for isolated ASC-exosomes. Stimulation of murine macrophage RAW264.7 cells with lipopolysaccharide (LPS) for 24 h substantially evoked production of nitric oxide (NO). However, co-treatment of ASC-exosomes significantly attenuated NO production in a dose-dependent manner (Additional file 1: Figure S2). Notably, the effect of ASC-exosomes was comparable to that of dexamethasone.

To investigate whether ASC-exosomes ameliorate AD symptoms in vivo, we evaluated the effects of ASC-exosomes in a murine model. AD-like lesions were induced by Biostir®-AD cream, which contains antigens from house dust mite, in NC/Nga mice (Additional file 1: Materials and methods) and ASC-exosomes were administered either intravenously (IV) or subcutaneously (SC) thrice a week for 4 weeks (Fig. 1a). As a positive control, prednisolone (10 mg/kg) was orally administered daily. We found that both IV and SC administration of ASC-exosomes significantly decreased AD symptoms in a dose-dependent manner (Fig. 1b, c). Consistently, ear thickness was also reduced in ASC-exosome-treated mice (Fig. 1b, d). We also found that the number of infiltrated mast cells was significantly reduced by ASC-exosome administration (Fig. 1b, e and Additional file 1: Figure S4). Additionally, the numbers of CD86+ and CD206+ cells decreased in the skin lesions after ASC-exosome administration (Fig. 1b, f, g, Additional file 1: Figures S5 and S6). Interestingly, it has been reported that inflammatory dendritic epidermal cells (IDECs), which are not found in normal skin but are abundant in AD skin, express both CD86 and CD206 on their surfaces [7, 8].

Since the elevation of the serum IgE level correlates with the severity of AD [9], we examined the effects of ASC-exosomes on the serum IgE level. The results showed that serum IgE levels were markedly reduced after ASC-exosome administration, either IV or SC, in a dose-dependent manner and these effects were comparable to that of prednisolone (Fig. 2a). As IgE mediates activation of mast cells and eosinophils [9], lowering serum IgE level by ASC-exosomes might result in reductions in mast cell infiltration (Fig. 1e) and the number of eosinophils. As expected, ASC-exosomes significantly lowered the number of eosinophils (Fig. 2b) but had little or no effect on neutrophil or white blood cell numbers (Additional file 1: Figure S3).

The mRNA levels of inflammatory cytokines were analyzed by quantitative real-time PCR (qRT-PCR). Interestingly, systemic administration of ASC-exosomes dose-dependently reduced the up-regulated mRNA levels of IL-4, IL-31, IL-23, and TNF-α in the skin lesions compared to vehicle control; the reduction was comparable to that with prednisolone treatment (Fig. 2c–f). In fact, all these pro-inflammatory cytokines are targets for biologics currently being developed or recently approved [1]. Downregulation of these multiple targets are well correlated with alleviation of AD symptoms in this study since IL-4 initiates isotype class switching to IgE and activates eosinophils [10]; IL-31 influences isotype class switching to IgE and recruits inflammatory cells into the skin and its level correlates with severity of AD [11]; IL-23 induces the differentiation of naïve T cells into highly pathogenic helper T cells that produce TNF-α [12]; and the plasma concentration of TNF-α is correlated with the severity of AD [13]. Collectively, these data demonstrate that systemic administration of ASC-exosomes ameliorates AD-like symptoms through the regulation of inflammatory responses and expression of inflammatory cytokines. These findings indicate that ASC-exosomes could be a novel cell-free therapeutic strategy to treat AD.

Despite the relevance of our findings, a limitation of the study is that potential donor variability remains to be addressed. In fact, a report has shown that donor age negatively impacts immuno-modulatory properties of ASC [14]. Further studies will be needed to confirm whether the potency of ASC-exosomes from aged donors correlates with our observations in younger, healthy donors.

Additional file

Additional file 1: Supporting information. (DOCX 6577 kb)

Abbreviations

AD: Atopic dermatitis; ASC: Adipose tissue-derived mesenchymal stem cell; BM: Bone marrow; IL: Interleukin; MSCs: Mesenchymal stem cells; qRT-PCR: Quantitative real-time PCR; TNF-α: Tumor necrosis factor-alpha; UCB: Umbilical cord blood

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Availability of data and materials

Data and reagents will be provided upon availability and reasonable request.

Authors' contributions

Conceived and designed the experiments: BSC and YWY. Performed the experiments: JOK, DHH, and YWY. Analyzed the data: BSC, JOK, DHH, and YWY. Wrote the paper: JOK and YWY. All authors read and approved the final manuscript.

Ethics approval

The animal study was approved by the Institutional Animal Care and Use Committee and performed in accordance with the Animal Experimentation Policy.

Consent for publication

Not applicable.

Competing interests

BSC and YWY are founders and stockholders of ExoCoBio Inc. All authors are employees of ExoCoBio Inc.

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cells

MDPI

Review

Advances in Regenerative Stem Cell Therapy in Androgenic Alopecia and Hair Loss: Wnt Pathway, Growth-Factor, and Mesenchymal Stem Cell Signaling Impact Analysis on Cell Growth and Hair Follicle Development

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Abstract: The use of stem cells has been reported to improve hair regrowth in several therapeutic strategies, including reversing the pathological mechanisms, that contribute to hair loss, regeneration of hair follicles, or creating hair using the tissue-engineering approach. Although various promising stem cell approaches are progressing via pre-clinical models to clinical trials, intraoperative stem cell treatments with a one-step procedure offer a quicker result by incorporating an autologous cell source without manipulation, which may be injected by surgeons through a well-established clinical practice. Many authors have concentrated on adipose-derived stromal vascular cells due to their ability to separate into numerous cell genealogies, platelet-rich plasma for its ability to enhance cell multiplication and neo-angiogenesis, as well as human follicle mesenchymal stem cells. In this paper, the significant improvements in intraoperative stem cell approaches, from in vivo models to clinical investigations, are reviewed. The potential regenerative instruments and functions of various cell populaces in the hair regrowth process are discussed. The addition of Wnt signaling in dermal papilla cells is considered a key factor in stimulating hair growth. Mesenchymal stem cell-derived signaling and growth factors obtained by platelets influence hair growth through cellular proliferation to prolong the anagen phase (FGF-7), induce cell growth (ERK activation), stimulate hair follicle development (β-catenin), and suppress apoptotic cues (Bcl-2 release and Akt activation).

Keywords: stem cell therapy; stem cell hair loss; human follicle stem cells; platelet-rich plasma; hair loss; hair regrowth; PRP hair; stem cells hair

1. Introduction

Hair tissue engineering and stem cell therapy are new approaches to treating hair loss (HL). Methods using exogenous cell sources or progenitor cells (PCs) are being tested in cell treatment clinical trials. These trials incorporate cells obtained from allogeneic and autologous sources. Specifically, intra-surgical cell treatments that incorporate autologous cell-based treatments with a one-step approach (cell harvesting, minimal manipulation, and immediate injection) into a single technique offer tremendous potential; a few methodologies have achieved clinical application. The intra-surgical cell treatment process involves tissue collection and preparation to obtain the desired cell product, followed by careful evaluation using the clinical application, and then cell conveyance. Intra-surgical

cell treatment benefits from the availability and safety of using the patient's own cells, which do not trigger an adverse reaction, as well as from the numerous important cell types that can be harvested using minimally invasive strategies [1].

This treatment bypasses a significant number of restrictions associated with exogenous cell treatment by avoiding in vitro cell control and expensive cell extension, the requirement for good manufacturing practice (GMP) facilities, the need to procure a work force for cell culture preparation, the potential for pollution, and a second method (at an alternate time point) to collect the cells. It might be helpful to maintain a strategic distance from the cell culture to restrict phenotype changes that may occur when cells are expelled from their local microenvironment for an all-encompassing time period [1].

Additionally, the techniques are entirely performed inside the surgical room (in absence of culture growth), which may lessen the hold-up time for the medical procedure. The U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and other administrative specialists consider grown-up cell products as biological products that can be partitioned into two classes: minimally manipulated biological products (obtained through centrifugation, filtration, and isolation without cell expansion) and manipulated biological products (obtained through culture-expanded stem cells). Certain intraoperative cell approaches fit the minimally-manipulated biological product category in which broad clinical trials are not required, consequently speeding up potential interpretation to facilities.

2. Hair Loss and Androgenic Alopecia: Bio-Molecular Pathway Disorder

HL is determined by an assortment of factors: inherited (trichodystrophy and androgenetic alopecia), accompanying comorbidity conditions, hormonal clutters (thyroid organ disease, insulin resistance), immune system (patchy alopecia and lupus erythematous), nutritional scatters, environmental elements (drugs, ultraviolet (UV) radiation), mental disorders (stress and trichotillomania), and aging. Harming factors influence the hair cycle (HC) and reduce stem cell activity and hair follicle recovery capacity [2].

A clinical need exists for the advancement of biotechnology to enhance hair growth to address the HL issue, specifically, in cases of androgenic alopecia (AGA).

AGA is a dynamic and incessant HL issue affecting 80% of white males and 40% of white females below the age of 70, in which lymphocytes and mast cells have been identified around the miniaturizing follicle in the stem-cell-rich knot zone [3–6]. The scaling down of the follicles (i.e., miniaturization) is characterized by a depletion of the anagen stage, with an enhancement in the measure of resting hair follicles (HF), telogen, and the presence of infinitesimal hairs on a bald scalp [5–7].

Current drugs available to treat AGA include medications such as Finasteride®; topical moisturizers such as Minoxidil®, and surgical procedures such as hair transplantation [4]. In HL scalps, the hair follicle stem cell numbers remain unaltered; however, the quantity of the more effectively multiplying PCs significantly decreases [7].

Along these lines, the aim of hair tissue engineering is developing new autologous advancements to induce hair regrowth by in vitro and ex vivo cultures or by in vivo recovery and bio-stimulation. Autologous stem cells (SCs) have been of particular interest for application in hair regrowth. Some early endeavors in the field concentrated on disengaging essential cells from a biopsy of the scalp tissue and maturing the cells ex vivo for a resulting injection into the patient.

Alopecia includes modifications to two sorts of hair SCs, represented by the hair follicle stem cells (HFSCs) and the dermal papilla cells (DPCs) [8,9].

HFSCs and DPCs guarantee conditions for appropriate hair recovery and regeneration [9]. In scarring alopecia (lupus erythematous, lichen planus), provocative cell invasion around the bulge results in an irreversible loss of HFSCs. Despite the PCs being harmed, HFSCs are saved in patchy and AGA, and this is the reason why this kind of HL can be reversible [9].

Bulge SCs have been progressively portrayed, particularly in murine HFs, thereby promoting their recognition, although no widespread marker has been found for them. An example is cytokeratin 15 (CK15), which explains why CK15+/integrin α6+ or CD34+/integrin α6+ cells have been distinguished as bulge cells [10].

Research on murine HFs has shown the expression of CK19 [11,12] and various transcriptional factors, that is, Gli1, Sox9, LHX2, Hopx, Tcf3, and Nfatc1, [11,13]. The expression of specific markers relies on the HC stage and on the exact area of the cells inside the bulge [11,14]. Lgr5, a receptor engaged in the Wnt signaling pathway, has been distinguished as a genuine marker of HFSCs [13].

The SCs of the upper and lower parts of the bulge in telogen HFs influence the expression of CD34 and part of the lower portion of Lgr5. Cells that are involved in the growth of another anagen hair express Lgr5 and not CD34 [15].

Cells of the upper piece of the bulge present a higher expression of Nfatc1, which is related to a condition of rest [6]. Expression of Lgr6 [14,16] and Lrig1 [14,17] has been observed inside the isthmus. The PCs of the germinal matrix are obtained from the SCs of the bulge, but unlike them, the PCs display a high grade of P-cadherin [11,18].

Human HFSCs (H-HFSCs) are less known than murine HFSCs (M-HFSCs). It appears that specific markers are normal in both human and mouse HFSCs: CD34 [19,20], K15 [8,19], K19 [19,21], and CD200 [8,19,20]. The presence of different markers (i.e., Sox9 and LHX2) requires further examination [22]. Markers found only in H-HFSCs are PHLDA1 [19,23] and EpCAM/Ber-EP4, which is considered a valuable marker of the telogen optional hair germ [18,19]. Dermal papilla (DP) cells present distinctive markers, including the cells from hair follicle cells (HFs) and dermal fibroblasts [24]. Alkaline phosphatase (ALP) is critical for both human and murine HFs, and it is the most explicit of the markers [21,22,24]; wherein its high action is considered a marker of DP cell separation [24,25]. Additionally, α-SMA [24,25], laminin, ad fibronectin [24], and CD133 [24,26] expression have been observed in DPCs.

Marker expression modify in disease states. The immunoreactivity of CK15 is diminished in individuals with patchy alopecia, and is also identified in AGA [10]. HF of the scalp's front part displays a shortage of CD34 in AGA, whereas its appearance is conserved in HF of the occipital locale [10]. In patchy alopecia, CD200, another marker of matrix cells, is expressed ineffectively, which might be an indication of a reduction in the immune benefit contributing to pathogenesis (i.e., response of auto reactive lymphocytes) [10,27].

SCs in the bulge remain in the resting stage for the vast majority of their lives, yet they can be actuated by relying upon the HC stage. Several theories with respect to the course and control of the HC have been proposed amid research in mouse models [12]. From the HC in mice, in the anagen stage, SCs in the bulge are separated multiple times and remain inside the niche, whereas cells of the germinal matrix divide strongly and produce the maturing hair shaft. During the catagen stage, cells from the germinal matrix experience apoptosis, while SCs of the bulge move out of it to the outer HF and, in this manner, toward the finish of the catagen stage, where they shape another bulge around the hair stem and another germinal matrix under the bulge. SCs in the bulge remain in a condition of rest during the telogen stage, and between the telogen and anagen stages, where they self-recover or move, creating a pool of germinal framework cells that multiply to shape the hair matrix [28].

The priority is on derivative cells in the bulge, which are the alleged SCs progenitor cells of the germinal matrix in the outflow of genes that influence stem cell activation, which have priority in expansion during the recovery cycle, even before the cells in the bulge [11,29,30].

The interpretation of the HC into the human HC has a few constraints due to the distinctive lengths of anagen [31,32], asynchrony of the human cycle [31,33], or the alternate response to the impact of hormonal variables [31,34]. Different papers reported the results of human scalp skin xenografted onto immune-compromised mice to set up the HC course in vivo in people [31,35].

The action of SCs in the bulge is controlled by its microenvironment (i.e., a supposed niche). This microenvironment incorporates the daughter cells of the SCs in the bulge, which enact their

self-recovery ahead of schedule and in the late anagen stages [36]. SCs are fundamentally influenced by the mesenchymal cells (MCs) of the DP, which are in close contact with the cells of the germinal matrix that are isolated by the basal membrane [14]. They appear to be of vital significance in the activation of hair growth and in signal transmission during recovery [11,30].

Investigations have demonstrated that hair recovery is impossible after laser treatment, on the grounds that the HF cycle stops at the telogen stage without advancing to the anagen stage [14,28,30,37]. Infusions of exosomes obtained from DPCs to HFs have been found to accelerate the passage of anagen and catagen delay by means of the β-catenin and Shh pathways [38]. HFSCs are additionally influenced by fibroblasts in the reticular and papillary layers of the dermis, in addition to the subcutaneous tissue [14]. Inside the niche, there are melanocyte SCs that are in charge of the formation of mature melanocytes that confer color to maturing hair. The survival and growth of MSCs relies on signs transmitted by the HF epithelial cells (ECs), for instance, the TGF-β or the Wnt pathway [14,36]. The extracellular matrix is another part of the microenvironment. It specifically influences the SCs through the arrangement of the basal layer, in which undifferentiated cells are in contact and regulated, for instance, by integrins [11,28].

3. SCs Use in HF Regeneration

HFs are immunologically-favored spots, similar to the cerebrum, eyes, and gonads, and they are influenced by the neuroendocrine-immune system [26]. In physiological conditions, this is influenced by: (1) Low expression or non-appearance of the principle MHC I antigens, (2) the presence of malfunctioning Langerhans cells, and (3) local expression of immunosuppressive substances (TGF-β1 and α-melanocytes MSH) [26,39]. Inferred from this is that HFs can be effortlessly used in transplantation.

Multipotent SCs can re-generate HFs with sebaceous organs in the skin. Given current information, SCs can be used to regenerate hairs in a few therapeutic methodologies such as: (1) Reversing the pathological mechanisms that determine HL (particularly in AGA), (2) regenerating mature HFs from their parts (cells in the bulge can regenerate an entire hair), and (3) neogenesis of HFs from a SC-culture with separated cells or tissue designing [40–42].

4. FDA and European Rules Regarding Use of Adipose Derived-Stromal Vascular Cells (AD-SVFs) and Human Follicle Mesenchymal Stem Cells (HF-MSCs)

The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) consider grown-up cell products as biological products that are isolated into two classes: minimally manipulated biological products and manipulated biological products. Regulation number 1394/2007 of the European Parliament for cutting-edge treatments defines "bioprocess engineering products," which excludes products that contain or are made solely of cells and non-vital human or animal tissues that do not have pharmacological, immunologic, or metabolic activity. Included amongst the advanced therapy pharmaceutical products are ones used for gene and somatic cell treatment (Directive 2001/83/European Parliament, Annex I). Cells and tissues are to be viewed as results of the bioprocess engineering products in the event that they experience "extensive manipulation". This rule contrasts between extensive and minimal manipulation. Manipulations that are not considered as bioprocess engineering include the following: cutting, granulating, forming, purification, centrifugation, absorbing anti-toxins or antimicrobial arrangements, cleansing, lighting, partition, fixation or decontamination, filtration, lyophilization, solidifying cryopreservation, and nitrification. The definition of medicines for advanced therapy excludes non-repetitive preparations completed under the supervision of a doctor running an individual remedy for a product explicitly intended for that specific patient, without obviously disregarding the important standards that identify with quality and security.

Further to the execution of Article 17 of Regulation (EC) No 1394/2007 (the Advanced Therapy Medicinal Products (ATMPs) Regulation), applicants are required to approach the Committee for Advanced Therapies (CAT) with a logical proposal for the arrangement of ATMPs. The committee is

in charge of surveying the quality, well-being, and viability of cutting-edge treatment medications, including medications delegated as quality treatment, substantial cell treatment, or tissue-built products. CAT is supported by the ATMP regulation, which empowers the EMA in a joint effort with the European Commission to decide if a given product meets the logical criteria that characterize ATMPs. The ATMP grouping technique was introduced with the goal of addressing inquiries into situations where the arrangement of a product dependent on genes, cells, or tissues is not clear. The CAT issues logical proposals to determine if the product falls within the definition of an ATMP in the European Union. The ATMP Regulation and Directive 2001/83/EC Annex I Part IV [30] provide exact legal definitions of ATMPs.

The ATMP characterization depends on an assessment of whether a given product satisfies one of the characteristics of gene therapy medicinal products (GTMP), somatic cell therapy medicinal products (sCTMPs), or tissue engineered products (TEPs), and whether that product satisfies the definition of a consolidated ATMP. It is additionally recognized that, because of the complex nature of these restorative products, the constrained information bundle at the beginning period of the product improvement, as well as the rapid growth of science and innovation, may result in inquiries off the fringe.

4.1. EMA/CAT Recommendations on Minimal Manipulation

According to the reflection paper on the portrayal of front-line treatment therapeutic products, EMA/CAT/600280/2010 Rev 1, June 20, 2014, by the Committee for Advanced Therapies (CAT), Line 10, "a similar basic capacity for a cell populace implies that the cells, when expelled from their unique condition in the human body are used to maintain the original capacity in a similar anatomical or histological condition." The authors presumed autologous application in a one-stage medical procedure, minimal manipulation, omofunctional use "used for an indistinguishable fundamental capacity in the beneficiary as in the donor", and manipulation with devices in aseptic conditions would be conditions that do not require good manufacturing practice (GMP) rules for preparation, good clinical practices (GCP) for the clinical application, or ethical committee underwriting.

4.2. Italian Rules Regarding Platelet-Rich Plasma Use

Platelet-rich plasma (PRP) preparation must be performed in Italy respecting the Decree of the Blood, November 2, 2015, dispositions related to quality, and safety parameters for blood and emocomponents.

5. Cell and Growth Factor Sources

Adult stem cells and PCs can be intra-surgically collected from a few tissues, including fat tissue, scalp tissue, bone marrow (BM), and peripheral blood. Previously, BM was viewed as a widely recognized source for these cells, since it was effortlessly and quickly obtained and because several devices were available for gathering BM. BM contains hematopoietic cells and MCs, as well as different cells types that may be involved in advancing tissue regeneration and hair regrowth. One of the primary constraints of intra-surgical SCs treatment approaches was the restricted amount of reaped source material and the low yield of cell detachment conventions, which yielded SCs at a frequency per mono-nucleated cell of one in every 10,000 cells to one in every 100,000 cells [43–46]. To overcome these difficulties and to avoid the highly invasive procedure of BM harvesting that causes pain at the donor area, additional elective sources from which to separate autologous SCs ought to be considered.

Fat tissue is an interesting source of cells that have multi-lineage separation potential. This tissue tends to be harvested using a less invasive technique, represented by liposuction and in larger amounts than in BM [47]. In fact, fat tissue harvest is much easier and less painful for the patient compared with BM gathering, in which it is necessary to use a trocar to drill the iliac crest [47]. A few investigations have reported different examinations and analyses between SCs obtained from various sources [47–50]. Peng et al. [47] included an immunophenotypic investigation of SCs separated from rodent BM and fat

tissue, and uncovered a noteworthy level of CD44+, CD73+, and CD90+ cells in fat tissue. Increased multiplication potential in cells detached from fat tissue was proposed, citing a higher and steadier growth rate all through 10 generations, a lower populace-doubling time, the highest proportion of cell populace in the S stage, and higher telomerase activity. These outcomes were confirmed by other examinations that emphasized the evaluation of various grownup, undifferentiated cell sources [48,50]. Fat tissue must be viewed as a real alternative to BM for intra-surgical use based on SCs wealth and expansion potential. A few patients may have constrained or minimal fat tissue for autologous settings, given the high frequency of adipose tissue-derived MSCs (their occurrence is 100 to 300 times higher than in bone marrow, and the number of SCs that can be counted per unit volume of fat harvested is approximately 10-fold greater than that from BM), small fat tissue repositories may still be adequate for SCs separation [48,51–53]. When gathering fat tissue for intraoperative SCs treatment, the tissue reaping site and the surgery affect the yield of undifferentiated cells. For example, fat tissue harvested from the abdominal region through resection or liposuction yields more SCs in contrast with ultrasound-assisted liposuction and with the fat tissue collected from the hip/thigh district [52].

For scalp tissue, in the preliminary examination performed by Cole J.P. et al. [53], they developed another system to separate human adult SCs with minimal manipulation depending on the centrifugation of fragments of human HFs without extension or culture. Specifically, the authors reported, for the first time, the results obtained in a hair regrowth study using a therapeutic device called Rigeneracons® (CE confirmed Class I, Human Brain Wave, Turin, Italy) that was used to obtain autologous micrografts containing HF-MSCs from centrifugation of a punch biopsy of the scalp, easily accessible for use in patients affected by AGA. The micrograft units were obtained by the disaggregation of a 2 mm punch biopsy by selection of a cell populace with a diameter of 50 microns. High cell viability was reported [53]. Noteworthy limitations include the challenge in growing cells to adequate numbers for human use, the need to conduct this expansion in good manufacturing practices (GMP) research centers, and the viability of the extended cells [54].

Therefore, the clinical use of HF-MSCs to enhance hair regrowth has not been satisfactorily considered. In other works [40,53], authors cited the amount of CD44+ cells (hair follicle-determined mesenchymal SCs) from the DP, and the level of CD200+ cells (hair follicle epithelial-SCs) from the bulge, obtained by means of the customized centrifugation of 11 punch tests [40,53]. The authors reported the microscopic evaluation of punch biopsy samples, performed using cytospin, immunocytochemistry, and the histological examination achieved by hematoxylin and eosin staining and clinical appraisal, where they discussed improvements to the current systems available for the recovery and regeneration of hair follicles. The authors emphasized permitting neo-genesis of HFs in adult individuals using isolated cells and biotechnologies [53].

The use of growth factors in autologous platelets was determined to provide substantial help in hair tissue regeneration owing to the platelets' ability to advance neo-angiogenesis, cell expansion, and separation [55–57]. Platelet-rich plasma (PRP) contains no less than six fundamental growth factors, including the fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and insulin-like growth factor-1 (IGF-1) discharged after platelet actuation [58]. Every one of these significant growth factors is engaged in an explicit bio-molecular activity.

Bio-Molecular Pathway of Stem Cells and Growth Factors That Improve Hair Regrowth

Hair regrowth was regulated prevalently by the Wnt biomolecular pathway and ERK activation in which SCs and growth factors are involved as reported following:

- Hepatocyte growth factor (HGF) and HGF activator (discharged by DPC) enhance the proliferation of follicular ECs;
- EGF improves the activity and growth of follicle outer-root sheath cells by activation of Wnt/β-catenin flagging;
- b-FGF improves the advancement of hair follicles;

- Interleukein-6 (IL-6) is involved in WIHN through STAT3 enactment;
- VEGF improves peri-follicular angiogenesis;
- TGF-β stimulates the signaling pathways that manage the HC;
- IGF-1 improves the migration, survival, and proliferation of HF cells;
- IGFBP-1 to -6 manages the IGF-1 effect and its connection with extracellular matrix proteins at the HF level;
- BMP maintains the DPC phenotype (fundamental for stimulation of HFSCs);
- BMPR1a maintains the proper identity of the DPCs (basic for explicit DPC work);
- M-CSF is involved in wound-induced hair growth;
- M-CSFR is involved in wound-induced hair growth;
- PDGF and PDGFR-β/-α64 up-regulate the genes associated with HF separation, induction, and control of anagen. PDGF and its receptors are fundamental for follicular improvement;
- Wnt3a is involved in HF advancement through β-catenin flagging;
- PGE2 stimulates anagen in HF;
- PGF2α and analogs enhance change from telogen to anagen;
- BIO (GSK-3 inhibitor);
- PGE2 or hindrance of PGD2 or PGD2 receptor D2/GPR4477 enhances follicle regeneration; and
- Iron and l-lysine95 (still under examination).

6. Clinical Intra-Surgical Applications of AD-SVFs in Hair Loss and Androgenic Alopecia

Another field involves the possibility of using fat and stromal vascular fraction cells (SVFs) for hair regrowth. SVFs are a heterogeneous gathering of non-cultured cells that can be constantly isolated from fat using minimal manipulation, using centrifugation, filtration, and the purification of fat tissue (proposed by EMA-CAT), or using enzymatic digestion (not proposed in the EMA-CAT suggestions). These cells work through paracrine frameworks to improve adipocyte suitability. Festa et al. [59] reported that adipocyte ancestry cells support stem cell function and help to drive the hair growth cycle. This follicular regenerative methodology is incipient and raises the probability that the HC in male and female HL can be driven or restored using autologous fat enhanced with SVF. Perez-Meza et al. [60] reported the safety and tolerability of these fat graft procedures in patients affected by inherited alopecia treated with sub-cutaneous scalp injections of fat tissue. An augmentation of 31.0 hairs/cm^2 was recorded in patients receiving a treatment of fat enriched with SVFs, where one subject who received fat alone (without SVFs addition) revealed a mean improvement of 14.0 hairs/cm^2, suggesting that fat grafts could be a treatment method for early HL, where the fat enhanced with SVFs may improve this response [60]. These studies suggest that scalp SVF-enhanced fat grafts could be a promising elective approach to treating HL in individuals. Fukuoka et al. [61] investigated the effects of fat-derived stem cell-conditioned medium injection in a gathering of 22 patients affected by AGA. Patients received treatment every three to five weeks for a total of six sessions. The mean augmentation in the hair number (hairs/0.65 cm^2) was 29 ± 4.1 in men and 15.6 ± 4.2 in women. No significative difference was observed between men and women.

An examination of the studies suggests that HF neo-genesis using human ECs and dermal cells is an extremely cumbersome process that requires novel culture conditions, somehow replicating the conventional or embryonic skin conditions and the use of embryonic or neonatal cells. There are no tissue regeneration protocols used in hair transplants involving the use of AD-SVFs. Zanzoterra et al. [62] analyzed the capacities of cell solution in the Rigenera® (Human Brain Wave, Turin, Italy) framework, which was obtained by the mechanical fracture of subcutaneous and fat tissue from the occipital region. The cell solution was injected into the hair transplant region, thereby expanding the measure of growth factors. Microscopic damage was seen to resolve more rapidly and the transplanted hair matured constantly, even two months after the strategy, wherein the telogen stage was abbreviated [62].

AD-SVFs-conditioned medium (AD-SVFs-CM) was used for patients with HL in an examination by Fukuoka and Suga [63]. A commercial product containing a protein arrangement with AD-SVFs was used (AAPE, Prostemics, Seoul, Korea), which had different growth factors (HGF, FGF-I, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor (GM-CSF), IL-6, VEGF, and TGF). The treatment (0.02 mL/cm^2 of the arrangement) was performed by injecting the suspension intradermal every three to five weeks (for four to six sessions), and hair growth was checked using trichograms. A real enhancement in hair thickness was accomplished in patients of both sexes [63]. Shin et al. [64] used AD-SVFs and the conditioned media of AD-SVFs (AD-SVFs-CM) in an observational examination of 27 women with female pattern hair loss (FPHL). The use of AD-SVFs-CM demonstrated efficacy in treating FPHL following 12 weeks of treatment, citing improvements in hair thickness and hair density without serious side effects [64]. Won et al. [65] also demonstrated that the use of AD-SVFs-CM upgraded the multiplication of cultured human DPCs by up to 130% [65].

Different examinations confirmed that enhancing fat tissue with SVFs supports adipocyte viability and yields better outcomes in a hair transplant treatment when they are applied in grafts [66,67].

6.1. AD-SVFs Regenerative Mechanisms

Recognizing the components that intervene in the impacts of intraoperative fat stem cell treatments is a problematic task. Current information that provides significant determinations or conclusions is lacking. This is not surprising as most of the fat SCs treatment evaluations focus on the appraisal of security and feasibility, and generally disregard the examinations aimed at understanding the mechanisms responsible for the results. Potential mechanisms involved are: (1) SCs or PCs inside the transplant renew the PCs in the host; (2) cells in the transplant differentiate and deliver new tissue; (3) transplanted cells emit trophic, angiogenic, or immunomodulatory factors that provide signals to nearby endogenous cells or distant cells, in the first case by means of paracrine signals, or in the second case (distant cells) by means of the endocrine mechanism (which may result in the assembly and homing of distant host cells); and (4) it is additionally conceivable that transplanted cells may combine with host cells (in a procedure known as cell combination).

The explanation of the mechanisms of the functional improvements is convoluted by the heterogeneous aspect of a significant number of the intra-surgically-injected cell populaces, where certain cell types may play a predominant role or various cell types may cooperate synergistically. In particular, as numerous intra-surgical cell treatment approaches use heterogeneous cell populaces that may incorporate stem or PCs, it is conceivable that positive results may not be mediated by the stem or PCs. Likewise, as cells are detached and handled inside the operating room, there is likely extensive variation in how the cells are acquired, prepared, and mixed (regardless of endeavors to institutionalize methods with all-around structured packs and equipment). This variance may affect the method of activity and results. Intraoperative cell treatments include disconnection of autologous cells from their source, resulting in noteworthy contrasts in the relative cell numbers and phenotypes, which may be derived from the different age, sex, race, weight list, family/hereditary genes, diet, and condition of the donors. The mechanism also relies on the type of infusion (e.g., manual versus mechanical and controlled) and isolation technique (e.g., enzymatic versus centrifugation and filtration). Along these lines, numerous inquiries remain unanswered, and new strategies and in vivo studies will be required to explain how transplanted cells mediate restorative reactions [68]. Almost certainly, the systems and mechanisms used to control the destiny and capacity of cells following transplantation (e.g., by designing exogenous cells inside the operating room) [69] will provide a line to follow to amplify the restorative impact. These techniques should help lessen the variability of the treatment response.

6.2. Adipose Tissue, Adipocyte, and AD-SVFs Potential Roles in Hair Loss

HFSCs are additionally influenced by the overall macro-environment encompassing the HF and fat tissue. Adipose tissue appears to experience comparative changes compared to the HF. The thickness of the fat tissue increases during the anagen stage and the adipocytes multiply greatly [11,70].

Adipocytes emit BMP2 during the late catagen stage and early telogen stage, which supports the resting states in the niche, whereas emission of BMP2 is lessened toward the finish of the telogen stage, which bolsters the activation of HF-MSCs [11,70,71].

Correspondence between fat tissue and the epithelium keeps running in the same line. Transformations hindering the HC have been found to restrain adipogenesis, which suggests that epithelium cells send signals actuating the expansion of the adipocytes [28,70]. The HFs absorb supplements from the micro-vascular network, which is changed amid the HC; angiogenesis expands during the anagen stage [28,71]. Bulge cells and the matrix may likely invigorate angiogenesis [28]. Delayed activation of angiogenesis, which is associated with impaired angiogenesis, has been observed in mice [14,71]. It has been recommended that SCs prefer low-oxygen conditions, where they emit markers of hypoxia [28,72].

The vascular system, particularly that encompassing the isthmus containing venous vessels, may participate in maintaining the low-oxygen conditions in the areas surrounding the SCs environment [28]. Although the impact of the immune reaction has not been adequately illustrated, it is critical that the job of maintaining the immune benefits of HF related to the diminished expression of MHC I molecules and to the improved discharge of immune-suppressors ought to be maintained during the anagen stage [28,35]. The loss of this benefit and a safe assault on cells of the matrix and the bulge are related to alopecia [28,39]. Dermal cells $\gamma\delta$T are known to regulate post-traumatic regeneration of HF by discharging FGF9 [14,73]. Therefore, macrophages increase the dimension of Wnt7b and Wnt10a ligands during the telogen stage after experiencing apoptosis, whereby it enacts HF-MSCs [14,28,74].

Macrophages play an essential role in the post-traumatic recruitment of HF-MSCs by arresting their enrollment into the injury postponing hair growth, although transplantation of dynamic macrophages is adequate for the enlistment of hair growth [28,75]. The job of Treg is also essential, wherein it introduces an abnormal state of Jag 1 from the Notch family, which influences the viable recovery of HF [76].

SVFs appear in a perfect cell populace for use in regenerative surgery due to the absence of immunogenic properties, their simplicity of acquisition, their multi-potential characteristic, the simplicity of separating them into different cell lines, and their significant potential for angiogenesis. SVFs have been created from wall cells situated in the perivascular portion, vascular smooth muscle cells, and pericytes—all associated with the arrangement of typical vasculature and are receptive to VEGF [77]. Normally, HFs encompassed by subcutaneous fat cells and by the dermis shape an inter-follicular dermal macro-environment, which is imperative for maintaining the best possible growth of bulge and follicle cells [24,59,78]. SVFs are vital for the activation of epidermal SCs, which they activate by discharging growth factors. The VEGF directs hair growth and the extent of the hair HF measure by stimulation of angiogenesis. HGF is associated with the span of the HC stages. The platelet-derived growth factor prompts and maintains the anagen stage, and IGF-I controls the hair growth cycle and hair cell separation [24,79]. Another heading for their activity is the stimulation of angiogenesis and enhancement of the blood supply to DPCs. Likewise, they have immunomodulatory and immunosuppressive properties via the collaboration among cells and the emission of prostaglandin E2 (PGE2), leukemia-inhibiting factor (LIF), and kynurenine [24,78].

The paracrine action of AD-SVFs is exceedingly complex, and the elements emitted by SCs have both a direct and an indirect impact on HFs. TB4 contributes to the activation of SCs in HF, improving their relocation into the follicle and separation. SDF-1 acts through an activation improvement of EGR-1, and it expands the cell tropism toward the follicle and builds angiogenesis. The activity of MCP-1, despite being an inflammatory factor, has a demonstrated tissue regenerative impact. A critical role of the microenvironment in the impact of paracrine factors in advancing the growth of the HF has also been underscored [78]. Huang et al. [80], in an investigation on rodents, found that an expansion of AD-SVFs to a culture of DPCs or core cells in the inner and external sheath improves their viability.

A huge increase in the regenerative potential was recorded in the investigation by Huang et al. [80], in which AD-SVFs were enhanced with LL-37, which is an antibacterial peptide normally present in

wounds. Their review demonstrated a significant improvement in the nearby regenerative factors (i.e., the endothelial growth factor, thymosin beta-4, monocyte chemo-attractant protein-1, and stromal cell-inferred factor-1). A significant improvement in the growth of HFs, in both in vitro and in vivo creature models, was also observed [77–81].

Physiologically, fat tissue encompassing HFs assumes a critical role in extending the anagen stage. Adipocytes PCs have been found to increase during the progress from the telogen to the anagen stage around the HF [59,79]. Unfortunately, two-dimensional (2D) cultures of DPCs lose their hair formation capability in culture, which is why they require maintenance of their spheroidal forms (3D) [82,83]. This is a challenge to mature methods that mimic in vivo conditions, which both maintain the 3D structure of the cells and contain a special medium, which imitates a natural niche rich in growth factors [84].

The increase in the thickness of the subcutaneous layer during the advanced hair growth stage (anagen) was contrasted with the thickness in the resting stage (telogen). SVFs invigorate HFs through peroxisome proliferator-activated receptors, in which three isoforms have been found on their surfaces (PPARα, PPARγ, and PPARδ) [79]. Mature adipocytes negatively affect HF cell expansion and the multiplication of fibroblasts encompassing the HF in concurrent culture frameworks [24,85].

Strangely, a change in the adipocyte cell line properties can cause skin and hair issues. Lipid disorders can cause deficits in the skin structure and function. Overexpression of human apolipoprotein C1 (APOC1) with hyperlipidemia in transgenic mice causes hair growth issues corresponding to the grade of expression of human APOC1 genes in the skin [24,86].

Hypoxia, which is not lethal to MCs, improves the generation of growth factors for the ADSCs: VEGF, PDGF, HGF, and IGF-II [84,87]. The impact of hypoxia on AD-SVFs was analyzed in an investigation by Park et al. [87], in which SVFs were passaged multiple times with CO_2 and subcutaneously injected into mice to observe induction of the anagen stage, and multiplication of human follicular cells of the DP and keratinocytes. Hypoxia produced a discharge increase in the insulin-like growth factor-restricting protein-1 and protein-2 (IGFBP), macrophage state invigorating element (M-CSF), M-CSF receptor, PDGF receptor-β, and VEGF, while the emission of the epidermal growth factor was lesser [87].

7. Clinical Intra-Surgical Application of PRP in HL and AGA

Many papers on PRP have been published, yet the outcomes are frequently conflicting. The authors take the perspective of not discussing PRP in general, though it is advisable to distinguish the different types of PRP relying on their cells content and fibrin design. Therefore, it is conceivable to recognize:

(1) Leukocyte-poor PRP (LP-PRP) or pure platelet-rich plasma (P-PRP): Suspension without leukocytes and with a low-density fibrin after induction;
(2) PRP and leukocyte (L-PRP): Suspensions with leukocytes and a low-density fibrin after induction (the largest of the commercial packages);
(3) Leukocyte-poor platelet-rich fibrin (LP-PRF) or pure platelet-rich fibrin (P-PRF): Suspension without leukocytes and a high-density fibrin;
(4) Leukocytes and platelet rich fibrin (L-PRF) or second-era PRP products are arrangements with leukocytes and a high fibrin density.

As discussed, there are too many protocols for the preparation of PRP depending on the different times for centrifugation and RPM used, the number of platelets, the accessibility of growth factors, and chemokines. Additionally, wide biological (among patients) and temporal (day-by-day) variation have been reported in the methods [88]. Thus, it is hard to evaluate which kind of PRP planning is better for clinical indications [89].

Diverse PRP products may be adequate for treating distinctive kinds of balding. The clinical efficacy of PRP is still under discussion, and a standardized protocol has not yet been created [90].

Doctors should choose the appropriate PRP preparations given their bio-molecular characteristics and clinical indications [91].

As of late, the use of low-level laser treatment (LLLT) has been proposed as a treatment for AGA and enhancing hair regrowth. The authors proposed LLLT 15 days after each treatment to stimulate hair regrowth during the HF-MSCs and PRP treatment, and every three weeks after the last treatment for a period of six months. Regarding this field, 11 papers were reviewed by Afifi et al. [92], where nine papers assessing hair count/hair density found noteworthy improvements in the men and women following LLLT treatment. Hair thickness and rigidity were found to be improved in two papers. Patient satisfaction was also accounted for in five of the works.

Autologous platelet-rich plasma (A-PRP) is now connected with enhanced surgical results and lower repeat rates when used in the gingival retreat and keloid treatments, respectively [93,94]. In dermatological uses, differences were discovered when PRP treatments were performed with the activated autologous PRP (AA-PRP) instead of the non-activated A-PRP. At the point when A-PRP is used with autologous thrombin to yield AA-PRP, healing of bone exposure [95], chronic injuries as severe hidradenitis suppurativa [96] and shorter recuperation times were observed for profound burns [97,98]. Similarly, laser use for acne produces subjectively better outcomes with fewer reactions when performed in combination with either topical or intradermal use of calcium-activated PRP [99]. These outcomes might be ascribed to the discharge and concentrations of alpha-granule proteins, including growth factors and cytokines, that stimulate cell separation and proliferation, angiogenesis, and vascular modeling [100].

In the treatment of HL, the topical use of AA-PRP with the collected follicles preceding implantation has been shown to build their survival rate by 15% [101]. Patients treated with calcium gluconate-initiated PRP displayed expanded hair thickness three months post-medical procedure, with terminal hair thickness (measurement > 40 μm) increasing by 19% during that time [102]. These discoveries were affirmed in an examination of AGA patients treated with calcium-activated PRP over a span of one year [103]. After 12 weeks from the last infusion of PRP, hair thickness reached a 19% expansion over the baseline estimations. At the one-year point, hair thickness decreased to 7% above the standard estimation, although this was still established as a significant increase in hair thickness in contrast to the baseline esteems [103].

The growth factors (GFs) acquired by the degranulation of the alpha-granules appear to stimulate hair regrowth. In detail, IGF-1 stimulates the multiplication of cycling Ki67+ basal keratinocytes [104,105], whereas TGF-β1 secures the proliferative capability of basal keratinocytes by repressing cell growth and terminal separation [106,107]. PDGF-AA increases the hair inductive action of DPCs when used in combination with fibroblast growth factor 2 (FGF-2) [108,109]. VEGF stimulates angiogenesis, and PDGF-BB is a strong chemo-attractant for wound macrophages and fibroblasts by stimulating these cells to discharge endogenous growth factors, including TGF-β1, which advances new collagen synthesis [110].

DPCs harvested from the human scalp have shown improved proliferation, improved Bcl-2 and FGF-7 levels, activated ERK and Akt proteins, and up-regulation of β-catenin when cultured in an initiated PRP-enhanced growth medium [111]. Each of these elements decidedly impacts hair growth through cell multiplication to prolong the anagen stage (FGF-7) [29], stimulating cell growth (ERK enactment) [112], invigorating HF development (β-catenin) [113], and stifling apoptotic signals (Bcl-2 discharge and Akt actuation) [114,115]. The human scalp affected by AGA treated with PRP injections should show significant increases in cell activity. Histological examinations of A-PRP- and AA-PRP-treated scalps from our past work [116] provide such clinical proof. In both patient populaces, the authors observed an enhancement in the number of follicular bulge cells and follicles, epidermal thickening, enhanced vascularization, and a higher number of Ki67+ basal keratinocytes in the PRP-treated scalp tissue compared to the placebo.

Hair regrowth in a clinical evaluation demonstrated a positive reaction to treatment with A-PRP in patients showing significative improvements in hair density and hair count in the treated zone

over the control zone (treated with the placebo). Differences between the 12-week follow-up hair counts and the baseline hair counts were observed. These hair growth parameters were higher in the A-PRP treatment group than in the AA-PRP treatment populace, as reported in past preliminary data reported by Gentile et al. [116]. Specifically, three-month hair density estimations for patients treated with A-PRP and AA-PRP were 65 ± 5 and 28 ± 4 hairs/cm^2, respectively. The outcomes established a 31 ± 2% improvement in hair density when the A-PRP treatment was performed versus a 19 ± 3% improvement in hair density when the AA-PRP treatment was performed, with a significant difference in hair growth (p = 0.0029). The increase in the hair growth parameters for A-PRP over AA-PRP may mirror the proficiency of in vivo thrombin in activating platelets and the body to distribute the contents of the activated platelets compared to in vitro calcium activation and infusion. The delivery of A-PRP may empower the production of thromboxane A2 (TXA2) by the platelets once they are activated in vivo, which would activate additional platelets and amplify platelet aggregation [117].

8. Clinical Intra-Surgical Application of HFSCs in Hair Loss and Androgenic Alopecia

It is hard to find particular strategies to enhance the regeneration of HF under conditions suitable for an adult individual. Given the knowledge on ECs and dermal cells, and their relationship in the midst of embryonic hair age and adult hair cycling, various scientists have tried to obtain mature hair follicles using techniques and procedures that rely on the causes for AGA [42,118]. In a preliminary examination [53], the authors developed another procedure to separate HFSCs using minimal manipulation, depending on the centrifugation of pieces of human hair follicles without cell expansion or enzymatic digestion. They reported the tallying of these cells and the preliminary results obtained from injections of micrografts containing HFSCs in the scalps of patients affected by AGA showed improvements in hair density. Gentile et al. [53] reported the amount of CD44+ cells from DP and the level of CD200+ cells from the bulge obtained using the customized centrifugation of 11 punch tests [53]. They also reported the microscopic evaluation of punch biopsy samples, determined by cytospin and immunocytochemistry, histological examination using hematoxylin and eosin staining, and clinical appraisal. The authors now seek to discuss improvements to the current systems available for the recovery and regeneration of hair follicles, focusing on systems permitting neo-genesis of hair follicles in adult individuals by using isolated cells and biotechnologies [53].

Examinations were performed using rodent cells, particularly of embryonic or infant origin. No fruitful procedure to produce human hair follicles from adult cells has been found. Possibly, the most crucial point is creating 3D culture conditions reflecting the structure of living tissue. It is necessary to improve the culture conditions that allow the expansion of specific cells while preserving their inductive properties, as well as procedures for picking masses of epithelial stem cells (ESCs), which should provide the principal instruments to overcome the difficulties constraining human HF neo-genesis [42]. These cells give the impression of being arranged in the bulge district of human hair follicles.

Hair Follicles and HF-MSCs Regenerative Mechanisms in Hair Loss and Androgenic Alopecia

HFs are known to have a well-characterized niche for grown-up SCs—the bulge, which contains ESCs and melanocytic SCs [119]. SCs in the hair bulge, an obviously-differentiated compartment inside the lower portion of hair follicles, can produce inter-follicular epidermis, HF structures, and sebaceous glands [120,121]. The bulge ESCs can also reconstitute in a simulated in vivo framework to a new HF [122,123].

Yu et al. [119] showed that follicles of human hair contain a SC populace that can be identified in the smooth muscle cell, as well as neuron and melanocyte heredities in the induction medium. Their analysis demonstrated that Oct4+ cells are present in human skin, and the greater proportion are positioned in the HFs in vivo. Oct4 has a place in the family of POU-domain transcription factors that are regularly communicated in the pluripotent cells of the developing embryo and that mediate pluripotency [124]. Additionally, human HFs contain multi-potent SCs other than ESCs and

melanocytic SCs, and these cells are positioned in the bulge region. These cells indicate promising plasticity in ex vivo and in vitro conditions, making them potential candidates for cell engineering and cell substitution treatments [124].

Each mature HF is a regenerating framework that physiologically experiences cycles of growth (anagen), relapse (catagen), and rest (telogen) at various times in an adult's life [125]. In catagen, HFSCs are maintained in the bulge. At that point, the resting follicle re-enters anagen (regeneration) when legitimate molecular signals are provided. During late telogen to early anagen change, signals from the DP stimulate the hair germ and quiescent bulge SCs to activate [29]. Numerous paracrine components are involved in this crosstalk at various HC stages, and some signaling pathways are involved [126–128]. In anagen, SCs in the bulge produce an ascent in hair germs, and at that point, the transient increasing cells in the grid of the new follicle proliferate rapidly to frame another hair filament [129]. The authors felt the need to better understand in which stage it is necessary to act.

Regeneration of HFs was observed in humans [130] when dermal sheath tissue was used, which was adequate to regenerate the DP structure. After implantation, the whisker DP was equipped to promote HF regeneration as holding the data to decide hair fiber type and follicle size [131]. In an examination [42], the authors prepared a dermal–epidermal skin substitute in a research facility by seeding an acellular dermal grid with cultured HF-ESCs and DPCs, both obtained from an adult human scalp. These constructs were grafted onto a full-thickness wound produced on bare mice skin. In 14 days, histological structures reminiscent of a wide range of phases of embryonic HF improvement were seen in the grafted region. These structures demonstrated concentric cellular layers of human origin and expressed k6hf, a keratin present in the epithelial cells of the companion layer. Despite completely mature hair follicles not being observed, these outcomes demonstrated that both epithelial- and dermal-cultured cells from the adult human scalp in a dermal scaffold could create in vivo structures that reiterate embryonic hair improvement.

Kalabusheva et al. [132] combined postnatal human DPCs and skin epidermal keratinocytes (KCs) in a hanging drop culture to mature a recreated HF germ. The procedure relied on DP cell hair-affecting properties and KC self-affiliation. The authors examined two protocols of aggregate gathering. Blended HF germ-like structures demonstrated the initiation of epithelial–mesenchymal cooperation, including Wnt pathway establishment and expression of follicular markers. They analyzed the effect of conceivable DP cell parts, including dissolvable segments and extracellular matrix (ECM) molecules during the time spent on the organoid collection and growth. Their results demonstrated that dissolvable parts had limited impact on HF germ age and Ki67+ cell scores inside the organoids, despite BMP6 and VD3 viably maintaining the DP character in the monolayer culture. Aggrecan, biglycan, fibronectin, and hyaluronic acid (HA) altogether improved cell multiplication in the DP cell monolayer culture with no effect on the DP cell character. A substantial part of ECM compounds confined the growth of cell aggregates, while HA propelled the formation of greater organoids.

Talavera-Adame et al. [133] uncovered the bio-molecular pathway involved in cell treatment. In particular, they exhibited that Wnt/β-catenin signaling was central to the growth and upkeep of DPCs [134,135]. The augmentation of Wnt signaling in DPCs is an essential factor that upgrades hair regrowth [134]. Specifically, in Pirastu et al. [136], androgen receptor flagging was involved in seven genes at six loci. Three primary gatherings were discovered: genes connected to Wnt flagging (*RSPO2*, *LGR4*, *WNT10A*, *WNT3*, *DKK2*, *SOX13*, *TWIST2*, *TWIST1*; *IQGAP1*, and *PRKD1*), genes involved in apoptosis (*DFFA*, *BCL2*, *IRF4*, *TOP1*, and *MAPT*), and heterogeneous gathering, including the androgen's receptor and TGF-beta pathways (*RUNX3*, *RUNX2*, *ALPL*, *PTHLH*, *RUNX1*, *AR*, *SRD5A2*, *PDGFA*, *PAX3*, and *FGF5*). Although a wide range of pathways have been implicated in the advancement of AGA, their outcomes suggest that, notwithstanding the androgen receptor pathway for which they affirm a fundamental function, the Wnt and apoptosis pathways assume a major role. AGA is described by shorter growth (anagen), which has been related to the increased apoptosis of the HFs. This outcome suggests that the anagen stage shortens as a result of contrasts in the genes managing the apoptosis. The Wnt pathway is involved in the advancement of telogen

(resting) to the anagen (growth), and in the fate of the SCs in the hair bulge, which are both dysregulated in balding tissue. Finally, hair loss chance loci in Wnt ligand biogenesis and trafficking and class B/2 (secretin family receptors) pathways were additionally connected with height, despite none of the individual loci in these pathways being crucial, suggesting a far-reaching impact. Along these lines, hairlessness indicates pathway-explicit hereditary relationships that provide a potential natural premise to the observed epidemiological connections. Pathway-specific hereditary relationships can help us to disentangle the mutually-organic pathways supporting complex pathologies [136].

9. Studies on Using Stem Cells from Wharton's Jelly

What are the advantages of using SCs from Wharton's jelly compared to other MCs? Wharton's jelly is a particular source of SCs due to its accessibility, vast pool of donors, non-invasive and easy harvesting, no hazard to the donor, no moral impediments, limited immunogenic potential, and high multi-potential separation ability [137,138]. In addition, the exposure to infectious agents seldom happens, which ensures security to the contributor [139]. The decellularized Wharton's jam matrix (DWJM) (fresh jelly exposed to two cycles of osmotic shock, alternately with a hypertonic suspension of NaCl, mannitol, $MgCl_2$, and KCl with an osmolarity of approx. 1.275 mOsm/L, centrifuged at 5000 rpm at 4 °C with a hypotonic arrangement of 0.005% Triton X-100) can provide a characteristic scaffold to SCs as a biocompatible matrix, which bolsters their viability, triggering conglomeration of MCs. DWJM contains TGF-β, collagen I, fibronectin, and tenascin, which might be in charge of condensing the added WJMSC in a few regions of the DWJM. In synthesis, DWJM is a natural biocompatible 3D matrix that guarantees adhesion, penetration, growth, and multiplication of cells, both in vitro and in vivo. To summarize, the report presented DWJM as a normal 3D scaffold that can be used in tissue designing and as a regenerative drug [140].

10. Concluding Remarks

Maintaining a pool of SCs is vital for tissue homeostasis and harm fix. Their divisions are not frequent in mature organisms, and their greater part are in a lethargic state. As such, it is vital to comprehend the components of their activation and induction, which will allow for the use of multi-potent cells in regenerative plastic surgery and hair regrowth. Their use is complicated by the fact that the expression of receptors on the various growth factors and the effect of the microenvironment may vary.

Not all target points in SC therapy have been distinguished. The information provided in this review features the useful impacts of AD-SVFs and PRP on hair regrowth, notwithstanding the preliminary positive outcomes from HF-MSCs. The activation and increase of Wnt signaling in DPCs is the crucial factor that enhances renewed hair growth.

The current knowledge in biology, the limits of past translational research that included development of pre-clinical and clinical models, harnessing of new strategies for more accurate imaging, and biomarker-based diagnostics will provide a strong basis to advance viable clinical approaches for regenerative aims in hair tissue engineering. Moreover, larger, randomized, double-blinded, controlled trials are needed to optimize cell administration protocols and to confirm the early observations of promising clinical outcomes.

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