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ARTICLE

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

Shadi Zari

Clinical, Cosmetic and Investigational Dermatology

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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

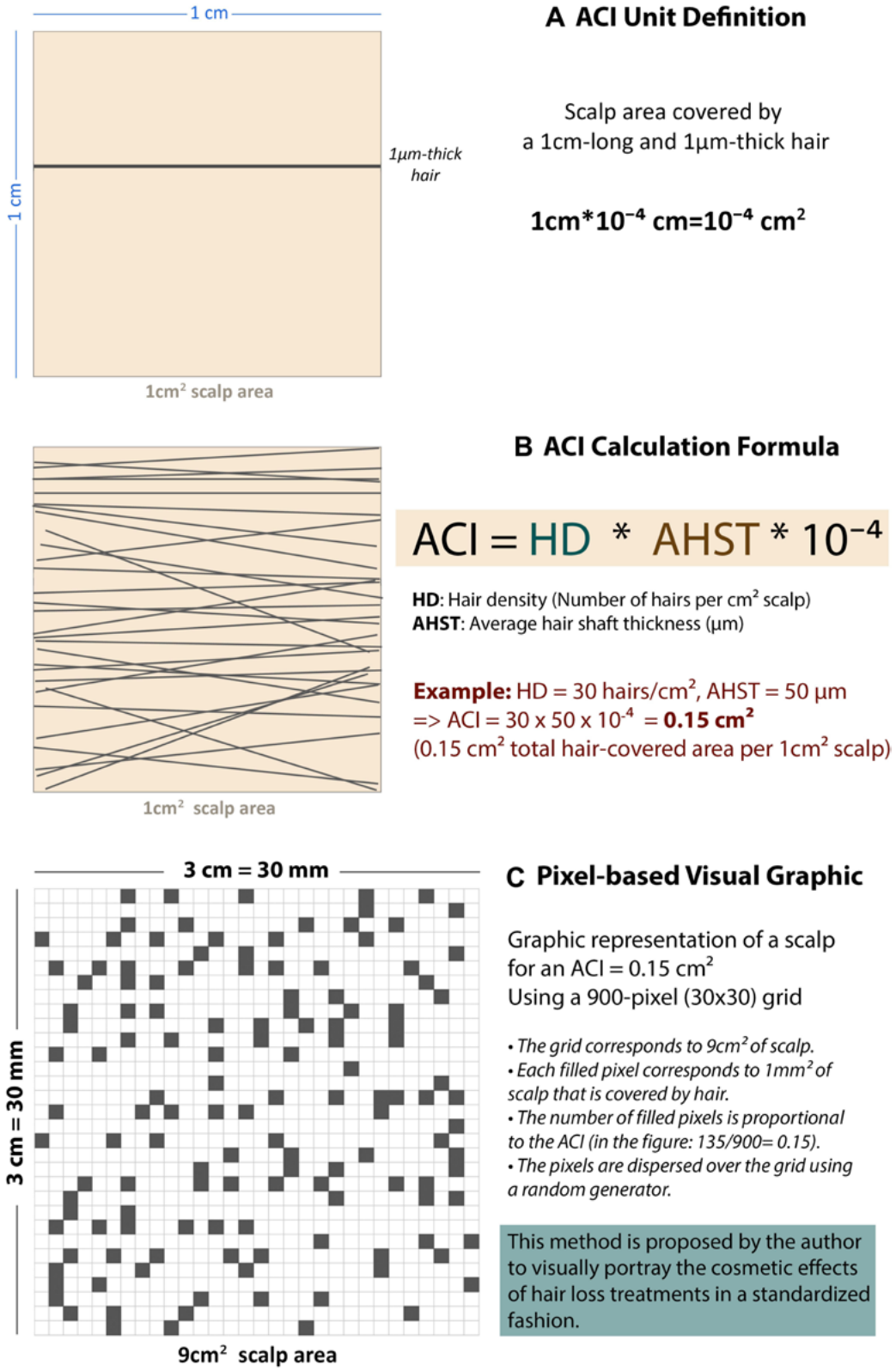

Figure 1 Area coverage index (ACI) definition, calculation formula and pixel transformation method. The figure describes the method proposed by the author to portray the visual aspect of the scalp as a function of the hair density and average hair thickness, by the (**A**) unit definition, (**B**) calculation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

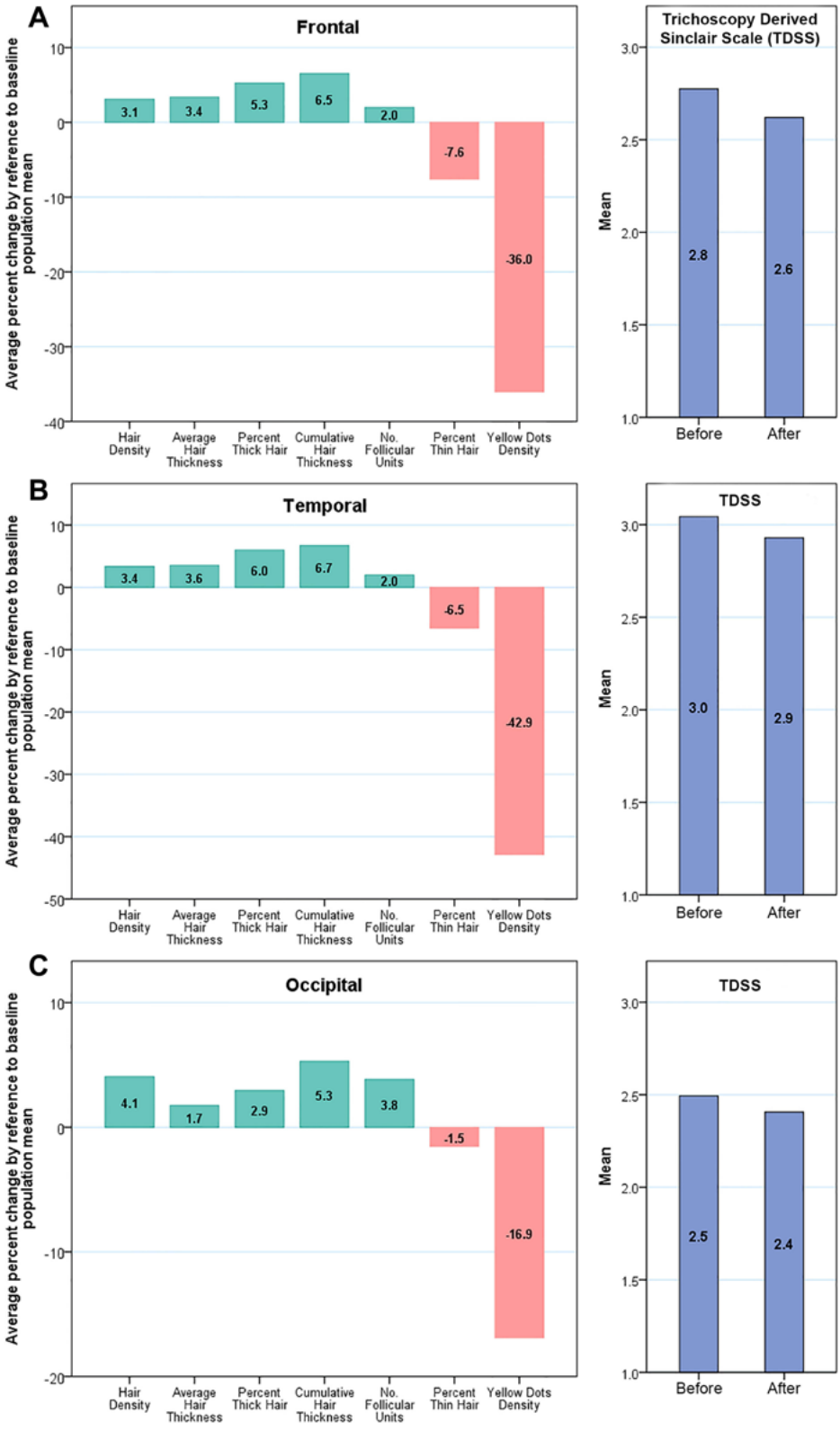

Figure 2 Change in key indicators of hair growth, by scalp region, in male and female androgenic alopecia after treatment with autologous cellular micrografts (ACM). Pictures depict: 1) relative pre- to post-intervention change (in percent, left panels) of the 5 positive (green bars) and 2 negative (Orange 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.

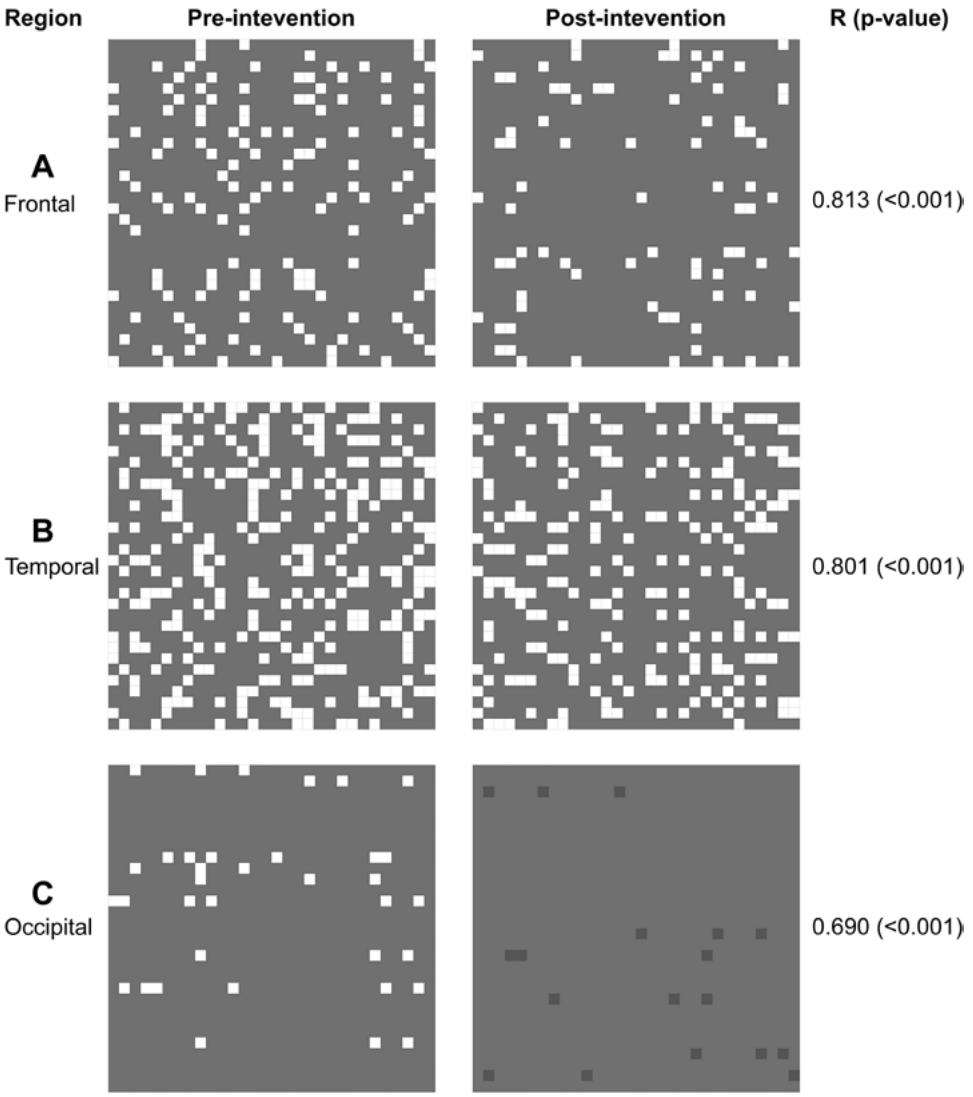

Figure 3 Short-term hair growth in male and female androgenic alopecia after treatment with autologous cellular micrografts (ACM). Pictures depict pre- to post-intervention change in area coverage index (ACI) in the frontal (**A**), temporal (**B**), and occipital (**C**) regions. Each grid represents 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

The author reports no conflicts of interest for this work and has no financial interests to disclose.

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02

ARTICLE

Clinical and Histological Evaluation of the Regenera® Method for the Treatment of Androgenetic Alopecia

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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).

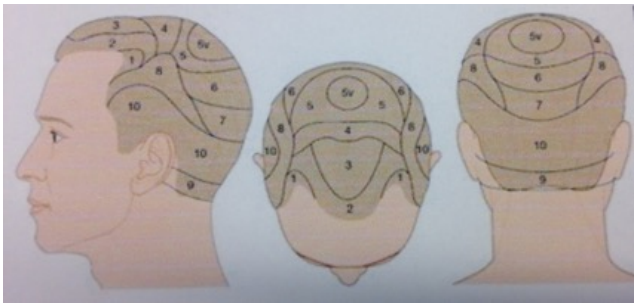

Figure 1: Application areas

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

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loss (47.05%).

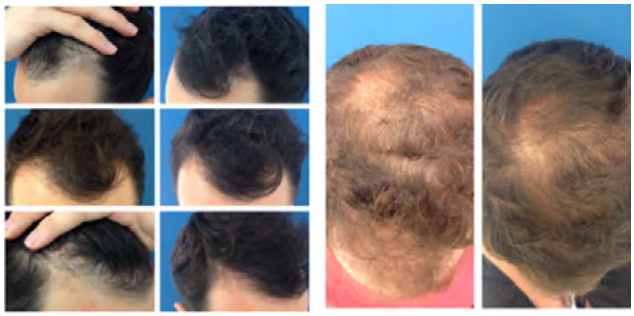

Figure 2: pre and post pictures. Left: case 1 (Day 0 top - Day 30 bottom). Right: case 2 (Day 0 left - Day 30 – right).

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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03

ARTICLE

Mesenchymal Stem Cells: Time to Change the Name!

Arnold I. Caplan

STEM CELLS TRANSLATIONAL MEDICINE®

PERSPECTIVES

Mesenchymal Stem Cells: Time to Change the Name!

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Key Words. Mesenchymal stem cells • Regenerative medicine • Medicinal signaling cells • MSCs

ABSTRACT

Mesenchymal stem cells (MSCs) were officially named more than 25 years ago to represent a class of cells from human and mammalian bone marrow and periosteum that could be isolated and expanded in culture while maintaining their in vitro capacity to be induced to form a variety of mesodermal phenotypes and tissues. The in vitro capacity to form bone, cartilage, fat, etc., became an assay for identifying this class of multipotent cells and around which several companies were formed in the 1990s to medically exploit the regenerative capabilities of MSCs. Today, there are hundreds of clinics and hundreds of clinical trials using human MSCs with very few, if any, focusing on the in vitro multipotential capacities of these cells. Unfortunately, the fact that MSCs are called "stem cells" is being used to infer that patients will receive direct medical benefit, because they imagine that these cells will differentiate into regenerating tissue-producing cells. Such a stem cell treatment will presumably cure the patient of their medically relevant difficulties ranging from osteoarthritic (bone-on-bone) knees to various neurological maladies including dementia. I now urge that we change the name of MSCs to Medicinal Signaling Cells to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors that are immunomodulatory and trophic (regenerative) meaning that these cells make therapeutic drugs in situ that are medicinal. It is, indeed, the patient's own site-specific and tissue-specific resident stem cells that construct the new tissue as stimulated by the bioactive factors secreted by the exogenously supplied MSCs. STEM CELLS TRANSLATIONAL MEDICINE *2017;00:000–000*

INTRODUCTION

Mesenchymal stem cells (MSCs) were officially named more than 25 years ago [1] to represent a class of cells from human [2] and mammalian bone marrow and periosteum [3] that could be isolated and expanded in culture while maintaining their in vitro capacity to be induced to form a variety of mesodermal phenotypes and tissues (Fig. 1, The Mesengenic Process). The in vitro capacity to form bone, cartilage, fat, etc., became an assay for identifying this class of multipotent cells [9] and around which several companies (including Osiris Therapeutics, which my colleagues and I started,) were formed in the 1990s to medically exploit the regenerative capabilities of MSCs. Initially, the driving concept that a multipotent progenitor or "stem cell" existed in adult marrow was not only challenged, but was actively disregarded, especially by the orthopedic industry. Fast-forward to today and there are hundreds of clinics [10] and hundreds of clinical trials [11] using human MSCs (hMSCs) with very few, if any, focusing on the in vitro multipotential capacities of these cells.

Unfortunately, the fact that MSCs are called "stem cells" is being used to infer that patients will receive direct medical benefit, because they imagine that these cells will differentiate into the regenerating tissue-producing cells (i.e., these "stem cells" will be incorporated into and these differentiated cells will fabricate the diseased or missing tissue). Such a stem cell treatment will presumably cure the patient of their medically relevant difficulties ranging from osteoarthritic (bone-on-bone) knees to various neurological maladies, including dementia. I long ago urged, in print, that we change the name of MSCs to Medicinal Signaling Cells [12] to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors [13] that are immunomodulatory and trophic [14] (regenerative), meaning that these cells make therapeutic drugs [15] that are medicinal. It is, indeed, the patient's own site-specific and tissue-specific resident stem cells that construct the new tissue as stimulated by the bioactive factors secreted by the exogenously supplied MSCs [16, 17].

HISTORY OF MSCS FROM A CAPLAN PERSPECTIVE

In the early 1970s into the 1980s, my colleagues and I published a number of papers based on the culturing of stage 24, embryonic chick limb bud mesodermal cells (ECLBMCs) that were observed to differentiate into cartilage, muscle, and bone under certain culture conditions [18–22]. These in vitro studies were correlated with a variety of in vivo studies that focused on the cellular and molecular events associated with the formation of embryonic limb bone [23, 24], cartilage [25], and muscle [26] in which several very prominent dogmas-of-the-day were challenged. For example, the concept that "cartilage is replaced by bone" led to the implication that if one could form cartilage in culture from embryonic mesodermal progenitor cells, one could observe the transition of that new cartilage into

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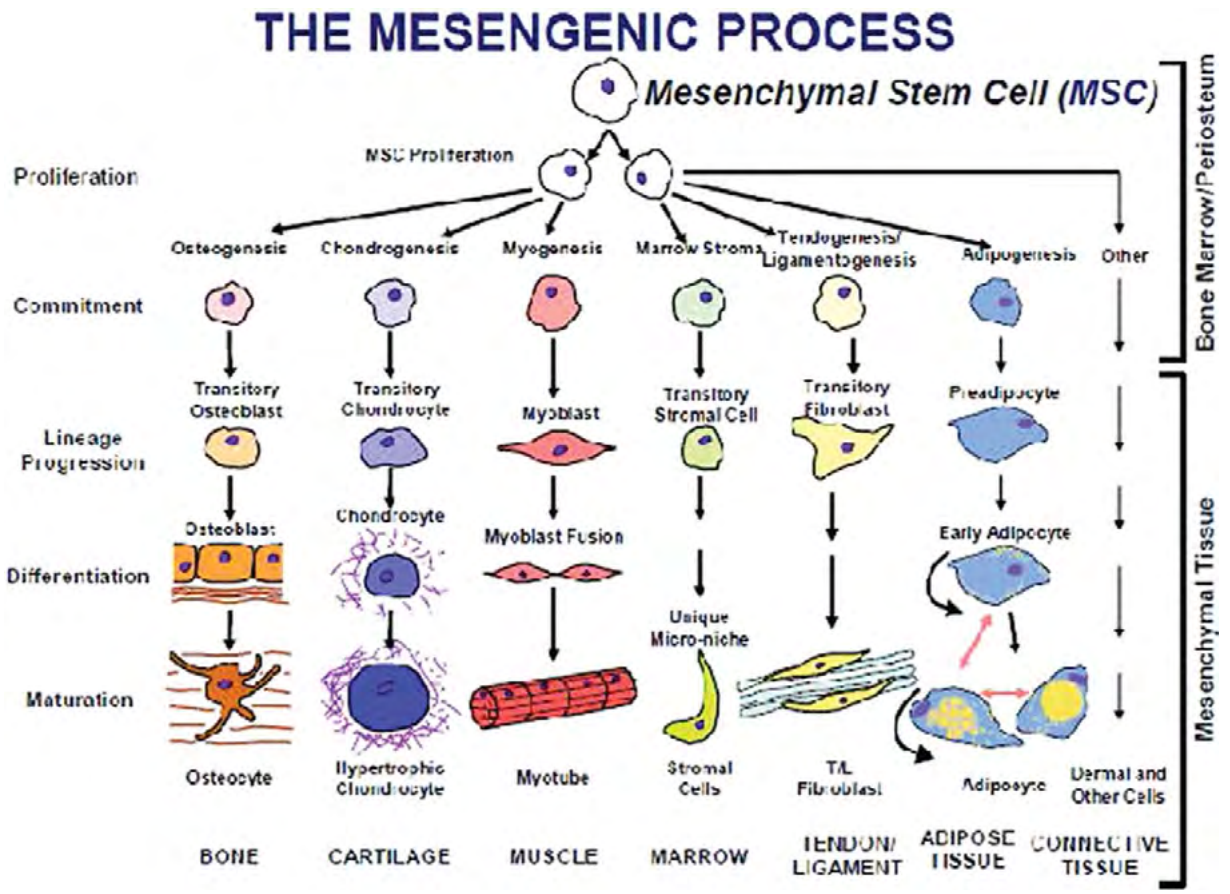

Figure 1. The mesengenic process. This hypothesis was originally verbalized in crude form in 1988 [4], refined as a figure in 1990 [5–7] and 1991 [1], with its current format published in 1994 [8]. All of the proposed lineage pathways to bone, cartilage, muscle, etc., have been verified by us and others using inductive cell culture conditions. There are no tissue-engineered products initiated with human MSCs that are approved and in use medically at this time. Abbreviation: MSC, mesenchymal stem cell.

bone. Moreover, the endochondral replacement of cartilage by bone implied that the cartilage so formed in culture would differentiate into hypertrophic cartilage, which would calcify and provide the calcified matrix for subsequent bone formation. Although we documented that the ECLBMCs formed cartilage in culture [22, 25, 26] and that hypertrophic chondrocytes could be identified by the production of type X collagen, the only mineral that formed in culture was observed in the noncartilage, connective tissue valleys between mounds of cartilage [23, 24]. Reducing the initial plating densities of the freshly isolated ECLBMCs (where no cartilage formed) allowed us to observe the differentiation of a maximum number of cells into calcified matrix-producing osteoblasts [27].

Only when we went back to the developing chick embryo and carefully completed rigorous histology of the mid-diaphysis of the developing embryonic tibia, did we firmly establish that the new bone that formed came from a progenitor cell layer (stacked cell layer) outside and away from the already formed and expanding cartilage core (or cartilage model as it was called [28]) [29, 30]. Importantly, the hypertrophic cartilage core was replaced by invading vasculature and then marrow, not bone. Moreover, others [31, 32] clearly showed that these embryonic hypertrophic chondrocytes could be isolated, cultured, and maintained for many weeks in vitro, documenting that hypertrophic chondrocytes were not "programmed" to die (i.e., their demise was due to the nutrient and oxygen deprivation by a collar of calcified bone that was outside and away from the cartilage core) [29].

In addition, our early pioneering studies on the synthesis of proteoglycans of cartilage by the ECLBMC cells in culture with Vincent Hascall's [33, 34] group brought us into areas of detailing the extracellular matrix (ECM) of first cartilage [35, 36], then muscle [37, 38] and bone and played an important role in our current interest in the basement membrane surrounding all blood vessels (to be discussed below).

We spent considerable time and effort in optimizing these stage 24 ECLBMC-cultures which, incidentally, we never called mesenchymal or mesodermal "stem cells," although the evidence strongly suggested that they were multipotent. During this same time period and especially in the early 1980s, Marshal Urist and others were isolating molecular agents from the matrix of demineralized bone [39–41]. The phenomenological basis for such efforts stemmed from implantations of demineralized bone pieces into muscle or subcutaneous pockets in rodents, which eventually caused bone to de novo form from host cells [39]. Urist coined the term "bone morphogenetic proteins (BMPs)" to summarily refer to the bioactive agents released from demineralized bone

matrix that could cause de novo bone to form in nonosseous tissues, such as muscle, or subcutaneously.

Stimulated by the public lectures and publications of Urist and because of a talented postdoctoral fellow, Glenn Syftestad, who had worked in Dr. Urist's lab at UCLA before coming to my lab in 1981, we joined the race to purify the BMPs. Our first approach was to take high salt extracts of demineralized bone exactly as published by Urist and to put them on cultures of stage 24 ECLBMCs arranged to just form bone [42, 43]. To our great surprise, these extracts caused the cells to form mounds of cartilage. We named the presumed active agents as chondrogenic stimulating activity, which we purified, and the university filed patents [44, 45], which for reasons that could be challenged, they stopped maintaining. In the mid-1980s and certainly by 1987, it became known that Dr. John Wozney and his colleagues at Genetics Institute, Inc. (Cambridge, MA) had cloned BMP2 and had patented the BMP-family of molecules [46, 47]. The race for the BMPs was over, and my colleagues and I had failed to win, much less "place or show."

In one of the demineralized bone implantation systems, Dr. Hari Reddi purified one member of this BMP-family and, importantly, characterized the in vivo temporal events caused by these factors [48–50]. These temporal events involved the invasion of the implant and cell division of host mesenchymal cells followed by their differentiation into cartilage which became hypertrophic and which was replaced by vascularized and marrowized bone [50]. Using Dr. Reddi's histology slides of these subcutaneous implantation specimens, which he generously provided, I suggested that the temporal sequence of cartilage replacement by bone was identical to that which we described in the developing embryonic chick tibia [5]—essentially, that the implanted demineralized bone particles were surrounded by mesenchymal progenitor cells, which were attracted to the demineralized particles and formed cartilage. Since the implant was walled-off, encysted by a layer of these mesenchymal cells comparable to the stack cell layer of the embryonic chick tibia, all blood vessels were excluded. The blood vessels outside the layer of surrounding and encysting mesenchymal cells caused the bottom layer of encircling cells to differentiate into a layer of osteoblasts, which fabricated a layer of osteoid that became mineralized. The deprivation of nutrients and oxygen caused the encased chondrocytes to form hypertrophic cartilage (Reddi documented the production of type X collagen) whose cells expired, releasing large quantities of vascular endothelial growth factor (VEGF), which caused the external vasculature to invade just as occurs in the mid-dyaphasis of the embryonic chick tibia [5, 29, 30]. These invading vessels brought a fresh supply of mesenchymal progenitor cells, which then formed vascularized and marrowized bone.

Without going into details, the central fact that comes from the above is that upon jamming the demineralized bone into muscle or the subcutaneous sites, the release and clustering of mesenchymal progenitor cells could be documented in these adult rodent hosts. In concert with these facts was the realization that adult bone marrow contained the same or similar primitive osteochondral progenitors [51–57]. The presence of these mesenchymal progenitors could be deduced from many avenues of exploration: (a) since the days of Aristotle, bone marrow was known to enhance orthopedic/bone healing [51]; (b) in modern terms, Connolly et al. [52, 53] and more recently Hernigou [58], documented the direct osteochondral potency of bone marrow or bone marrow aspirates; (c) Friedenstein et al., as rediscovered and popularized by Owen, showed that clones of adherent osteogenic progenitor cells could be isolated and propagated in culture from adult marrow [54–57]; and last (d) Owen herself imagined a crude mesenchymal lineage comparable to that described for descendants of hematopoietic stem cell (HSC) [57]. It is important to stress that in the 1980s and early 1990s, the dogma-of-the-day was that the only stem cells that existed in the adult body were HSCs.

The above facts (especially the demineralized bone implantations into adult hosts) led Dr. Stephen Haynesworth and me to see if we could isolate and purify the mesenchymal progenitor cells from adult human bone marrow [1, 59–63]. At that time, we were not aware of the work of Friedenstein and of Owen, which was lucky because we had the ECLBMC system, which was quite different from the culture conditions of Friedenstein and Owen. We had long before optimized this ECLBMC system, in particular by optimally choosing the batch of fetal bovine serum (FBS) used to culture these chick embryonic cells [64]. This lucky batch of serum was later shown to be optimal for the attachment, propagation, and maintenance of the multipotency of the culture adherent cells from human adult marrow [65, 66]. Indeed, one in 10–20 batches of FBS was shown to be optimal for marrow-derived hMSCs by the ECLBMC culture assay system, which eventually was replaced by other criteria [66]. This assayed batch of FBS allowed MSCs to optimally attach to the culture dishes, to expand to form colonies (referred to as colony form units/fibroblast, CFU-f by Friedenstein [55], that could be counted to give MSC titers, which ranged from 1 in 10,000 marrow cells in newborns to 1 in 2 million marrow cells in 80-year-old marrow donors [67]. Given all of the above, I named these propagated cells that were multipotent in culture assay: MSCs [1].

MSCs: Various Names Mean the Same

Given the historic outline above, various names for these culture adherent and passaged adult marrow-derived, multipotent mesenchymal cells came to mind:

Marrow Stromal Cells

The term "stroma" is an older morphological term meaning from connective tissue or the structural component of tissue. As defined by Owen in 1988 [57], these are fibroblastic cells that adhere to plastic and expand, forming colonies (CFU-f) that are osteogenic. One could also envision that bone marrow stroma was a unique scaffold that supports different lineage arms of hematopoiesis. Such a three-dimensional connective tissue scaffold does not exist in marrow, although the vision of such a specialized framework is enchanting.

Multipotent Stromal Cells

MSCs can be multipotent, as documented in various culture circumstances.

Mesodermal Stem Cells

Because of our studies of ECLBMC cells, this term was highly favored, especially because all of the induced or bioactive factor-treated cells and tissue formed in culture were of mesodermal (middle layer of the embryo) origin.

MSCs

I chose this term because mesenchyme is a type of tissue characterized by loosely associated cells that lack polarity and are surrounded by a large ECM. Because of their in vitro multipotency and clonability [68], I, provocatively, called them "stem cells" to especially appeal to the orthopedic community. As defined by hematologists, all stem cells must be capable of serial transplantation and unlimited doublings. Indeed, there are published reports that support this definition [69, 70].

Mesenchymal Stromal Cell

A group of scientists at an international meeting termed the MSC as a "stromal" cell because they did not favor the stem cell classification and imagined, incorrectly, that the origin of MSCs, from a variety of tissues, was the connective tissue layer of that tissue [9].

Medicinal Signaling Cell

Because the function of MSCs in vivo is secretory and primarily functional at sites of injury, disease, or inflammation, I now favor this terminology [12].

The New Science: MSCs Are Derived from Pericytes

Central to the renaming strategy is the fact that most, if not all, MSCs are derived from the differentiation of perivascular or mural cells, pericytes [71]. The studies of Dr. Bruno Péault and colleagues [72] clearly document that pericytes isolated from a variety of tissues give rise to MSCs, as identified by cell surface antigens and their in vitro multipotency. Importantly, MSCs can be isolated from every vascularized tissue [73] and even from menstrual flow [74, 75] (i.e., broken blood vessels release the perivascular cells that differentiate into MSCs). The perivascular location as the origin of MSCs and their functional capacity to be immunomodulatory and trophic (including fabricating and secreting antibiotic proteins [76]) challenges the "stromal" name and origin of the MSCs [9, 77, 78].

Based on the above, we have assembled the new and current information on the pericyte MSC (pMSC) into a poster, which has a number of interesting and unusual pieces of information not previously appreciated [79]. These include the fact that each separate tissue-specific stem cell is both in communication with its underlying vascular endothelial cells and neighboring specific pericyte/MSC [Universal Stem Cell Niche]. These pMSCs are specific to each stem cell, including a chemically different pMSC next to the active versus quiescent HSC in marrow [80, 81]. In every tissue examined in detail, the marrow, neural tissue [82], liver [83], heart [84], etc., tissue-specific stem cells are next to its specific pMSC on a blood vessel. These observations further support the concept that all pMSCs have both MSC-common and MSC-unique chemical and functional features. In the in vitro multipotency assays, the assay must be optimized for each tissue specific MSC. For example, hMSCs of marrow were shown to be induced in culture into the chondrogenic lineage by TGF-β [85], while fat-derived hMSCs require both TGF-β and BMP-6 [86]. The main in vivo functional differences of MSCs from different tissues or organs remain largely unknown, even though the major therapeutic functionality of MSCs at various sites of disease or injury are very similar when comparing these different MSCs [87].

Changing Names

Since the main functionality in vivo of MSCs [88] is not multipotency and, thus, not as a stem cell [89–91], I propose that its name be changed. The precedent for changing medical terms is not new. For example, names of many diseases have been changed: ablepsy was changed to blindness; ague to malarial fever; American plague to yellow fever; anasarca to generalized massive edema; aphonia to laryngitis; aphtha to thrush in infants; and apoplexy to paralysis due to stroke [92]. Of course, there is great stigma associated with the accepted names for some diseases; multiple sclerosis was once called hysterical paralysis when people believe this was caused by stress linked with oedipal fixations. Chronic fatigue syndrome is a serious ailment, yet 85% of clinicians view it as a psychiatric disorder; activists are currently trying to change the name to remove the bias and stigma. There is no stigma associated with the term MSC except, for me, the implied promise that it is a true "stem cell," which it is not in vivo.

It has been argued, because MSC science and clinical use is so strong and, indeed, positive with almost 700 clinical trials listed on clinicaltrials.gov, that the MSC nomenclature should remain. The problem is not with the "mesenchymal" part of the name; it is the "stem cell" part of the name that is the issue. As outlined in our poster, the pMSC functions quite differently from the released pericyte that forms an activated, site-specific MSC. Infused auto- or allogenic MSCs appear to home in on active vascular sites of injury or inflammation [93]. At such disease sites, the MSC rarely or never differentiate into the tissue at that site [13, 88], but they secrete bioactive factors (some of the names of these factors we know [94]) and their therapeutic effects can be analyzed as site-specific clinical outcome parameters. Outcomes for graft-versus-host disease, acute myocardial infarct, low back pain, osteoarthritic knees, tendonitis, and aspects of inflammatory bowel disease or Crohn's disease have been reported (www.mesoblast.com). Again, for emphasis, these MSC-effects are medicinal.

MSCs Are Not Stem Cells

The science and commercialization of adult MSCs were enhanced by the popularization of embryonic stem cells (ESCs) and made more attractive by President Bush's prohibition of the use and study of ESCs [95]. This popularization of ESCs also served as a disadvantage because all "stem cells" have been viewed by the public as being pluripotent or multipotent. Thus, the infusion of hMSCs in an osteoarthritic knee is imagined to contribute directly into the regeneration of cartilage tissue by the infused MSCs forming functional chondrocytes that fabricate functional cartilage tissue. The infusion into cardiac patients of hMSCs assumes that these cells will directly convert into functional heart muscle cells to replace the cells that die from the ischemia of the heart attack. And so on and so on: stem cells directly convert into the diseased or injured tissue in question. Although we, in this field, all have our own favorite explanation for the mechanisms that govern the observed positive therapeutic outcomes, the in vivo effects of infused hMSCs are best described as medicinal and most likely not associated with the infused cells differentiating into regenerative or replacement tissue [96–99].

These stem cell misconceptions have led some practitioners in the United States and worldwide to advertise the availability of stem cell-treatments (i.e., MSCs can cure the blind, make the lame walk, and make old tissue young again [10]). I, of course,

want the MSC nomenclature to remain in use, but not as stem cells. Perhaps we should call them magic signaling cells, more strategic cells, maxi secreting cells, most sensitive cells, main secreting cells, or message secreting cells. I propose to change the name of MSCs to reflect our new understanding that they do not function in the body as progenitors for tissues, neither in the normal steady-state nor in disease or injury circumstances; they are not stem cells.

MSCs and Metastasis

Last, we recently published a treatise which documents that the pMSC actively binds to and pulls circulating melanoma cells into the marrow of bone [100]. This grab/pull mechanism for melanoma metastasis is counter to the current concept that metastatic cells secrete digestive enzymes that allow the melanoma to erode its way into bone. We further hypothesized that the laminin identity in the basement membrane ECM of the blood vessels plays both an active and permissive role in the extravasation of melanoma into bone. Thus, the melanoma must pass through the endothelial cell layer, its basal lamina or basement membrane and past the dense covering of mural cells. The active pMSC not only facilitates this extravasation, but is actively and molecularly controlling this translocation from the circulation into the marrow of bone. Clearly, the pMSC is not medicinal in this context even though its differentiated progeny, the MSC, can provide powerful medicinal benefit given other circumstances. Last, the pMSC is not multipotent nor does it, itself, cause tumors to form. The pMSC is corrupted by the cancer cell; it does not corrupt normal cells to become cancerous.

Conclusion

It should be permissible for the person who named the MSCs to drop the stem cell nomenclature because it is scientifically and therapeutically misleading. In 2010, I proposed that we call them medicinal signaling cells [12]. That is what these do, and the culture plasticity of most mesenchymal cells (we can induce adult human chondrocytes to make a bone or fat in cell cultures [101]) means that the stem cell moniker is inappropriate. I was wrong. I take back the name that I gave these hugely important cells. Call them MSCs, but please, not stem cells.

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Disclosure of Potential Conflicts of Interest

Case Western Reserve University receives royalties from Osiris Therapeutics, Inc. (now by Mesoblast Limited) which they share with AIC for MSC technology transfered in 1992. No other conflicts exist.

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ARTICLE

The MSC: An Injury Drugstore

Arnold I. Caplan and Diego Correa

Cell Stem Cell
Perspective

The MSC: An Injury Drugstore

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Now that mesenchymal stem cells (MSCs) have been shown to be perivascular in vivo, the existing traditional view that focuses on the multipotent differentiation capacity of these cells should be expanded to include their equally interesting role as cellular modulators that brings them into a broader therapeutic scenario. We discuss existing evidence that leads us to propose that during local injury, MSCs are released from their perivascular location, become activated, and establish a regenerative microenvironment by secreting bioactive molecules and regulating the local immune response. These trophic and immunomodulatory activities suggest that MSCs may serve as site-regulated "drugstores" in vivo.

The Mesenchymal Stem Cell

In embryonic development, the mesodermal layer harbors multipotent progenitors that give rise to bone, cartilage, muscle, and other mesenchymal tissues. Based on this embryonic perspective and previous reports from our group and others, a hypothetical and comprehensive scheme, pictured in Figure 1, proposed that in adult bone marrow (BM), a population of mesenchymal stem cells (MSCs) could likewise give rise to a spectrum of mesenchymal tissues by differentiating along separate and distinct lineage pathways (Caplan, 1991). In the early iterations of this model, cells from the marrow were the main focus because orthopedic surgeons had long ago established that cells from this tissue could be used to stimulate bone formation and repair, and because it had been demonstrated that osteoprogenitor cells originated from BM (Friedenstein et al., 1966, 1987; Tavassoli and Crosby, 1968; Owen and Friedenstein, 1988). Using this same logic, we and then others successfully isolated and culture expanded MSCs from adult human BM and documented the multipotency for mesenchymal differentiation by these heterogeneous cell populations and by clones of these cells as predicted in the model pictured in Figure 1. This finding encouraged us and others to explore the use of MSCs as progenitors for use in tissue engineering to replace or repair damaged tissues of mesenchymal origin.

What we lost with this isolated focus on multipotency and tissue engineering was the question of what MSCs naturally do in BM and other tissues, and what intrinsic physiological roles these populations may play in vivo, beyond how their functional traits might be harnessed in response to artificial cues or settings. Indeed, it was recognized that MSCs can support hematopoiesis in culture, and this finding focused our attention on their potential to constitute the supportive BM stroma (Majumdar et al., 1998). With this capacity in mind, the first clinical trials conducted by our colleagues with culture-expanded MSCs were designed to augment and support BM transplantation (BMT) for cancer patients (Lazarus et al., 1995). Because of this focus on BMT and the aversion to the term "stem cells," others proposed (Horwitz et al., 2005) that MSCs be called "multipotential mesenchymal stromal cells," and sometimes just "marrow stromal cells," terms that keep the MSC abbreviation but, for us and some others, have always seemed to be inappropriate. The nomenclature issues, however, arise from the difficulties of reconciling in one term the fact that MSCs, at least from BM, do exhibit stemness properties, including self-renewal capacity under clonogenic conditions (Sacchetti et al., 2007; Dennis et al., 1999; Baksh et al., 2004; Bruder et al., 1997; Colter et al., 2000) and multipotential differentiation capabilities (Pittenger et al., 1999; Mackay et al., 1998; Dennis et al., 1999; Prockop, 1997; Giordano et al., 2007), once they are isolated from the nonparenchymal component ("stroma") of various tissues.

This logic would likewise apply to other tissue-derived cells, such as adipose-derived stem cells (ADSCs), which exhibit similar ex vivo multipotency (Rodeheffer et al., 2008; Tang et al., 2008). At least some of the current debate stems from the fact that while the multipotential capacity of MSCs has been proven in vitro (Pittenger et al., 1999), the in vivo counterpart is still not definitive. However, it is important to mention that in spite of the fact that multipotency should be strictly defined using clonogenic experiments, this single-cell approach does not necessarily reflect the in vivo situation. Additionally, the lack of an unambiguous in vivo MSC marker that identifies this cell population in different tissues highlights the possibility that different cell characteristics may be dictated by the local tissue microenvironment in which they reside (Bianco et al., 2008). This technical shortcoming also poses limitations when comparing the general performance of different isolated populations, given the inconsistencies often seen in terms of the isolation and characterization methods employed. To complicate the problem even further, the "pure" mesodermal origin of MSCs is still debatable, given the potential additional ectodermal origin through ectoderm-derived neural crest in craniofacial bones (Hall, 2008).

The Pericyte

As described above, the early studies of MSCs depended on their isolation, expansion, and characterization in vitro, and considerable effort has been expended toward identifying and localizing these cells in situ. There is a detailed and elegant literature (Hirschi and D'Amore, 1996; Crisan et al., 2008; Traktuev et al., 2008; Sacchetti et al., 2007) that supports the fact that for almost every blood vessel in the body, mesenchymal cells are observed in perivascular locations (on both arterial and venous vessels). These abluminal cells, called pericytes for

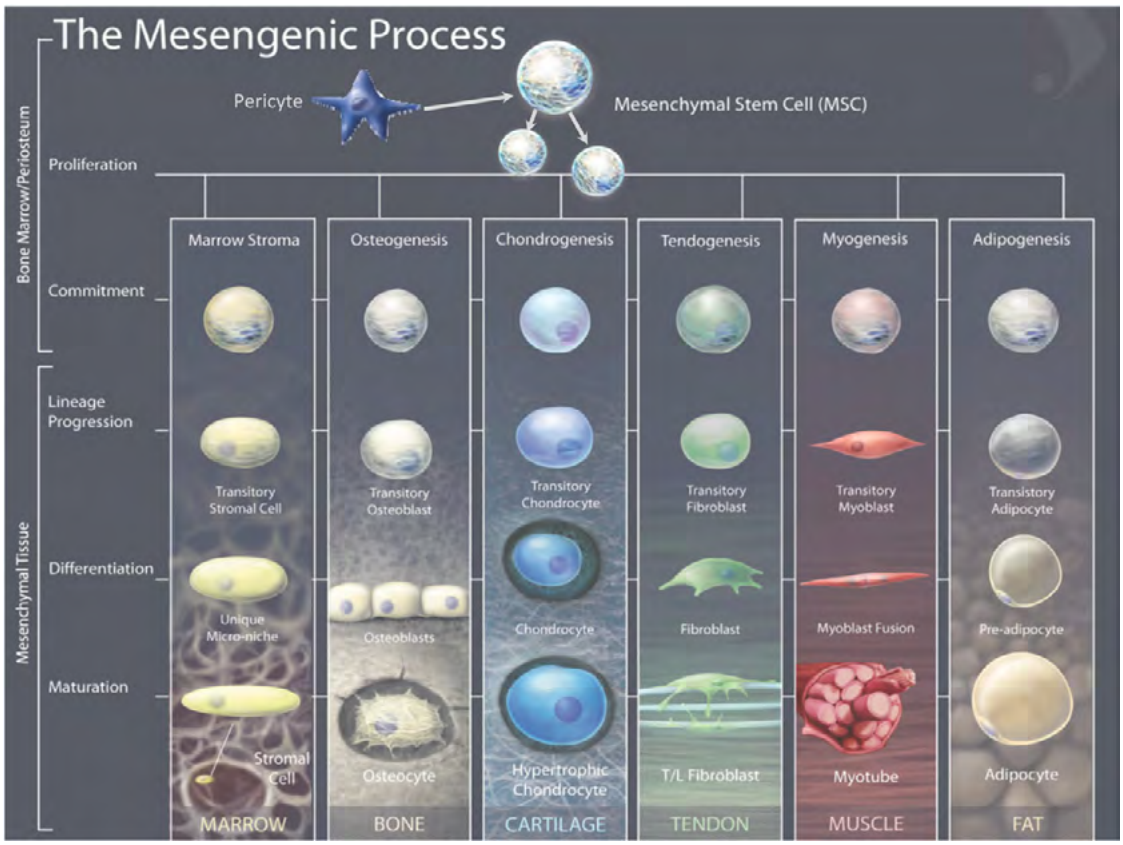

Figure 1. The Mesengenic Process

The original version of this figure was generated in the late 1980s (Caplan, 1991, 1994) and has been modernized in this rendition. The figure proposes that an MSC exists in the bone marrow and that its progeny can be induced to enter one of several mesenchymal lineage pathways. The lineage format was constructed from what was known about the hematopoietic lineage pathway, and this figure depicts the predicted differentiation hierarchy of the most prominent candidate lineages. (Current image graphics produced by Michael Gilkey, National Center for Regenerative Medicine).

convenience, are in intimate contact with the basement membrane and surrounding endothelial cells that comprise the microvasculature, from precapillary arterioles to small collecting venules. A continuum of phenotypic similarities is apparent across various vessel types in that pure pericytic cells are observed in the microvessels, while the smooth muscle cells that are typically present in terminal arterioles, venules, and larger vessels retain the expression of some pericytic markers such as NG2 and CD146 (Crisan et al., 2008; Díaz-Flores et al., 2009). It is now clear that isolated pericytes exhibit a panel of cell surface markers that are identical to those expressed by isolated MSCs (Crisan et al., 2008). Furthermore, as described in this issue of *Cell Stem Cell*, a novel cell surface-specific marker of ADSCs (WAT7, which corresponds to a cleavage product of decorin) is also expressed in vivo by perivascular cells that exhibit typical pericyte markers such as PDGFR-β and αSMA (Daquinag et al., 2011). These and other observations allowed us to speculate in a commentary in this journal that all MSCs are pericytes (Caplan, 2008). If most or all MSCs are indeed pericytes, it opens new possibilities regarding how to physiologically and therapeutically visualize the role of MSCs. In particular, if pericytes are the source of MSCs, do these cells have local functions in the tissue microenvironment beyond their mesenchymal differentiation capabilities?

Preclinical Animal Models and Clinical Trials

The potential therapeutic benefit of exogenous MSCs has been under preclinical investigation for many years. Between 1995 and 2011, both autologous and allogeneic MSCs from multiple sources have been injected into tissue sites such as heart or infused into the blood stream and have been observed to localize to tissue sites of injury involving broken or inflamed blood vessels. As of May 2011, the NIH website (http://clinicaltrials.gov) lists 19,364 cell-based therapies, and 206 of those are considered MSC-related. The list of MSC-related candidate applications includes diverse clinical targets, indications, or clinical conditions, such as BMT, graft versus host disease, acute myocardial infarct, stroke, spinal cord (cuts and contusions), lung (asthma and chronic obstructive pulmonary disease [COPD]), acute kidney failure, liver fibrosis, tendinitis, juvenile diabetes, radiation syndrome, burns and wound healing, osteoarthritis and rheumatoid arthritis, lupus, autism, inflammatory bowel disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), urinary incontinence, and sepsis. Consistent with the proposal that there is an ongoing change in philosophy with regard to the clinical potential offered by MSCs, almost all of these trials and preclinical models utilize MSCs in therapeutic and medicinal manners that are quite distinct from the capacity of the cells to differentiate into different phenotypic lineages.

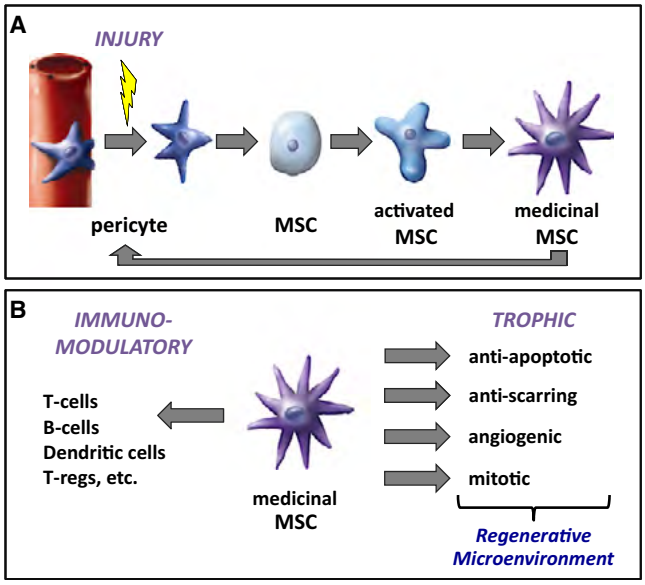

Figure 2. MSCs Are Immunomodulatory and Trophic
(A) The proposed sequential activation of pericytes as a response to injury. Local vessel damage affects resident pericytes and liberates them from functional contact with blood vessels to become activated MSCs. Upon immune activation, these mobilized, "medicinal" MSCs secrete factors that organize a regenerative microenvironment. Subsequent repair is reinforced when activated MSCs reacquire a stabilizing pericyte phenotype in the abluminal space. (B) The bioactive molecules secreted by medicinal MSCs are immunomodulatory and affect a variety of immune cell lineages (Aggarwal and Pittenger, 2005). Other secreted molecules establish a regenerative microenvironment by establishing a powerful trophic field (Caplan, 2010).

Indeed, all of these disorders and conditions appear to be muted or cured by the injected or infused MSCs based on two generalizable therapeutic activities (Caplan and Dennis, 2006): immunomodulation and trophic activities (Figure 2). The immunoactivity of these cells has been shown to be mediated by both secreted bioactive molecules and by cell-cell contact, and can involve dendritic cells and B and T cells, including T regulatory cells, killer cells, and a variety of T helper cells (Iyer and Rojas, 2008; Jones and McTaggart, 2008; Le Blanc et al., 2003). The trophic effects involve MSC-secreted molecules that inhibit apoptosis (especially caused by ischemia) and scar formation. They also involve stimulation of MSC-mediated angiogenesis by secretion of VEGF and by the MSC stabilization of new vessels by the return to their earlier pericyte phenotype (Sorrell et al., 2009). Lastly, MSC-secreted mitogens stimulate tissue-intrinsic progenitors to divide and appropriately differentiate (Wagner et al., 2009; Rehman et al., 2004). In this regard, we have published a compendium that identifies the molecular agents secreted by MSCs that contribute to these immunomodulatory and trophic effects (Meirelles Lda et al., 2009; Singer and Caplan, 2011).

The Drugstore

Based on the examples described above, we support the model that MSCs are clinically active at different tissue sites, that MSCs are pericytes and can be isolated from any vascularized tissue, and that MSCs secrete large quantities of a variety of bioactive molecules as part of their local trophic and immunomodulatory activities. We propose that this specific MSC tissue "regulatory" phenotype arises as a consequence of broken or inflamed blood vessels at sites of tissue damage. This model does not exclude the possibility that pericytes naturally have an on/off cycle in the noninjured situation. We envision that this active phenotype can be adopted in addition to their "constitutive" phenotype in which, as perivascular cells, this population expresses MSC markers both in vivo and ex vivo and functionally exhibits multipotential ex vivo differentiation capabilities (Crisan et al., 2008; Sacchetti et al., 2007; Pittenger et al., 1999; Mackay et al., 1998; Dennis et al., 1999; Prockop, 1997; Giordano et al., 2007). According to this paradigm, in situations of vessel damage, the released pericytes become MSCs, are activated by the injury, and respond to that tissue site by secreting a spectrum of bioactive molecules (i.e., drugs) that serve to, first, inhibit any immune cell coming to survey the tissue damage and, thus, prevent autoimmune activities from developing (Figure 2). In addition, these secreted bioactive molecules, through their trophic activities, establish a regenerative microenvironment to support the regeneration and refabrication of the injured tissue. In this context, the MSCs serve as site-regulated, multidrug dispensaries, or "drugstores," to promote and support the natural regeneration of focal injuries. If these injuries are large or occur in older individuals, the natural supply of MSCs must be supplemented by local or systemic delivery.

Although most existing clinical information has been generated to date using culture expanded marrow-derived MSCs, there is information to suggest that MSCs from fat, placenta, umbilical cord, and muscle have similar, but not identical, functional potential (Guilak et al., 2010; Moretti et al., 2010; Hass et al., 2011). However, the question of which tissue source of exogenously supplied MSCs might be optimal for a given clinical situation has not yet been established. What is quite clear, however, is that allogeneic human MSCs do not elicit a vigorous immune response that leads to their rejection even after multiple infusions (Aggarwal and Pittenger, 2005; Koç et al., 2002; Le Blanc et al., 2008; Ringdén et al., 2006). Moreover, we and others routinely utilize culture-expanded human MSCs from many human donors in animal models of disease (MS, asthma, inflammatory bowel disease, etc.) with reproducible efficacy (Bonfield et al., 2010; Bai et al., 2009). This pattern of clinical application does not question the potential efficacy of autologous MSCs, although one could envision that some autoimmune diseases might be initiated in response to a malfunction of these endogenous, resident MSCs should this population no longer provide adequate immunomodulation at the affected tissue site. Similarly, for select conditions, the provision of autologous MSCs might inadvertently exacerbate a targeted disease state, particularly if an autoimmune component is evident, for example, in MS. In this latter case, for the MSCs to be curative they must not only mute the inflammatory or autoimmune activity that causes demyelination, but they must stimulate the differentiation and site-specific functioning of oligodendrocytes from local progenitors to rewrap the denuded axons in the central nervous system as has been shown in animal models (Miller et al., 2010) .

Unanticipated Efficacy

The theme above centers on diseases where immunomodulation and trophic activities can affect the progression of the clinical

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presentation. Completely unanticipated is the recent publication that human MSCs also make a protein that is a lethal antimicrobial for both gram-positive and gram-negative bacteria. The synthesis of this cathelicidin by MSCs, called hCAP-18/LL37, was shown to be a dose-of-bacteria-dependent antimicrobial when tested in an intratracheally (IT)-instilled mouse model of *E. coli*-induced pneumonia (Krasnodembskaya et al., 2010). In this work, IT-delivered MSCs reduced the growth of bacteria and promoted their clearance from the animals, as evaluated by lung homogenates and bronchoalveolar lavages. These results may be applicable to other devastating lung infections, such as the ones present in cystic fibrosis (CF) patients, where IT or aerosolized cell preparations may have potential therapeutic benefits. The control of these tissue-specific bacterial infections exerted by MSCs can now be added to their known systemic bacterial growth control in different models of induced sepsis (Németh et al., 2009; Gonzalez-Rey et al., 2009). We further speculate that this MSC-dependent antimicrobial activity is normally present in the lung, oral cavity, gut, etc. Thus, MSCs may be extremely useful in both local and disseminated infections.

Medical Applications

Given all of the above, we envision that the clinical use of MSCs may change the course of the practice of medicine. For instance, based on the known effects of infused MSCs on heart diseases (Schuster et al., 2004; Itescu et al., 2003; Minguell and Erices, 2006), it may be possible to develop an alternative therapeutic paradigm for use in third world countries or in situations in which adequate life-support is not readily available for patients suffering an acute myocardial infarct. Following this new approach, patients would be treated in a clinical facility that had frozen stores of bags of allogeneic MSCs available for infusion. This early therapy, combined with subsequent support treatments that are already in current use, would be expected to stop the progression of myocardium loss and serve to limit and minimize the long-term effects of the cardiac ischemia. Therefore, the scientific advance in our understanding of the properties of MSCs as a potential therapy for heart diseases, and their subsequent potential for clinical application, raises a central and important question: How long must we wait for this therapy to become widely available, given that it is based on the cells serving as drugstores that dispense secreted trophic factors? Because the current proposed clinical uses of MSCs have nothing to do with their multipotency, we have suggested that we call MSCs *m*edicinal *s*ignaling *c*ells (Caplan, 2010). It should be noted that both neural stem cells and hematopoietic stem cells likewise have the capacity to secrete a diverse set of bioactive molecules that have both immunomodulatory and trophic activities. Thus, we are careful to say that these local pro-regenerative activities may not be directly related to the specific differentiation capacity of multipotent progenitors, but instead may be a common feature of adult stem cell populations.

The Lesson Learned

Perhaps the most important lesson learned from the past 20 years of MSC research is that we must continually ask what the native, normal functions of these cells are. Of course, this line of investigation is particularly challenging in the absence of tools that allow the identification and tracking of specific, homogeneous populations of MSCs in vivo. Scientists are enormously clever in terms of the tricks we can make cells perform in the context of manipulated culture conditions. However, how to translate these tricks into successful clinical protocols has proven to be elusive. The powerful, natural capacities of these isolated cells when put back into the body either as freshly harvested cells or after culture expansion is the more important discovery, and these clinical observations provide a window into understanding their normal physiology and normal cell function. This insight helps us design more informative and revealing experiments that will lead to the eventual translation of our science into practical and effective clinical treatments. Much work needs to be done to carefully define the clinical circumstances where MSCs should be utilized and to more precisely define their mode of action.

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ARTICLE

Adult Mesenchymal Stem Cells: When, Where, and How

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Review Article

Adult Mesenchymal Stem Cells: When, Where, and How

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Adult mesenchymal stem cells (MSCs) have profound medicinal effects at body sites of tissue injury, disease, or inflammation as either endogenously or exogenously supplied. The medicinal effects are either immunomodulatory or trophic or both. When to deliver these mediators of regeneration, where, and by what delivery apparatus or mechanism will directly determine their medical efficacy. The MSCs help manage the innate regenerative capacity of almost every body tissue and the MSCs have only recently been fully appreciated. Perhaps the most skilled physician-manager of the body's innate regenerative capacity is in orthopedics where the vigorous regeneration and repair capacity of bone through local MSCs-titers is expertly managed by the orthopaedic physician. The challenge is to extend MSCs expertise to address other tissue dysfunctions and diseases. The medicine of tomorrow will encompass optimizing the tissues' intrinsic regenerative potential through management of local MSCs.

1. Introduction

Since the late 1980s when the technology for isolating and culture expanding MSCs was perfected and then reduced to practice in the early 1990s [1, 2], their use for clinically relevant therapies has evolved. Indeed, two very different logics have been proposed and explored. The original logic was that marrow-derived, culture-expanded MSCs, because of their multipotency, could be used in tissue engineering formats to replace injured, damaged, or diseased mesenchymal tissues [3, 4]. Although this logic was pursued for almost three decades and continues to be explored, no product or treatment is currently available. In defense of this pursuit, newer logics and scaffolds now being experimentally tested hold realistic promise for eventual success and clinical use to replace cadaveric products now used routinely.

The documentation that MSCs (perhaps all MSCs) are derived from perivascular cells, pericytes [5, 6], now explains how MSCs can be isolated from almost every tissue in the body [7, 8]. Moreover, the fact that MSCs possess the capacity to secrete immunomodulatory and trophic mediators strongly argues that their natural and normal *in vivo* function is as Medicinal Signaling Cells (MSCs) for sites of injury or inflammation [9, 10] in all of the tissues in which they are housed. Today on the website *clinicaltrials.gov* a search using "mesenchymal stem cells" in the website's search engine shows that over 500+ clinical trials are listed covering a surprisingly enormous array of clinical conditions. All of these clinical conditions have one or both of the immunomodulatory or regenerative (trophic) aspects as central components to the therapeutic intent of using MSCs.

The focus of this treatise is to take the state of knowledge, at *this point in time*, to address the medicinal use of MSCs and to attempt to identify the key parameters to consider for their optimal use in cell-based therapies. In this context, some misconceptions will be addressed since the state of detailed knowledge is relatively small compared to the exuberant expectations of the physicians and scientists consumed by the therapeutic potential of MSCs, the present author included. Thus, this paper is a report on the state of the art of MSCs and it is expected that these new, powerful potential therapeutics will evolve as we have previously witnessed when considering the changes in use and science of hematopoietic and neural stem cells in the last 50 years of their clinical and experimental exploration [11, 12].

2. MSCs

The realization that MSCs are derived from pericytes changes the context of considering how they arise and function *in*

vivo during the body's response to both localized injury and the demand for regeneration/repair. In its simplest inception, the pericyte is released from its association with the basal lamina of the blood vessel situated in the field of injury or inflammation. This released pericyte is exquisitely capable of sensing its surrounding *milieu* and responding by becoming an MSC; this new MSC phenotype becomes activated and keyed to the detailed chemistry and dynamic changes to its local microenvironment. The activated MSCs put out a concentrated localized curtain of bioactive molecules that serves to inhibit the interrogating cells of the body's overaggressive immune system [13, 14]. This is, thus, a first-line defense against the establishment of autoimmune reactions against the injured tissue in the immediate vicinity. In coordination with this protective curtain, the MSC secretes molecules to assist in the establishment of a regenerative (not repair) microenvironment. Included in these trophic mediators are molecules that (A) inhibit ischemia-caused apoptosis; (B) inhibit scar formations; (C) stimulate angiogenesis and vessel stability; and (D) stimulate mitosis of tissue-intrinsic progenitors [15, 16].

The overall effect of locally situated, activated MSCs is to help manage the innate capacity of every tissue to regenerate itself by inhibiting the quick-fix apparatus of scar formation. It is now apparent that the immune system contributes components that not only protect injury sites from "foreign intruders" but also enhance the quick-fix aspects of fill-in with connective tissue that leads to scar. Clearly, in embryos where the immune system has not developed, scarless healing is quite normal [17]. Likewise, in neonates, the scarless regenerative capacity is substantial. As animals get larger and as they age, the vascular density in various tissues decreases and tissue regeneration, or even repair, becomes logistically different [18]. The key to the MSCs' clinical efficacy is the fact that every living tissue turns over. This means that as cellular and extracellular matrix (ECM) components expire, they are replaced by similar components. The innate regenerative capacity of a tissue is tied to this turnover dynamic. For example, the fact that bone is resorbed and fabricated in a coupled cellular mechanism allows fractures to heal at a rate directly linked to the natural, age-related ratio of fabrication to resorption, that is, rapid healing in young growing subjects and very slow healing in older, osteoporotic subjects [19, 20]. This begs the question of whether it is a youthful microenvironment (i.e., molecular) that controls turnover/repair or whether it is the cells themselves that provide the dynamic queuing.

If MSCs are, indeed, the managers of site-specific tissue regeneration, their presence, their numbers, their proper activation, and their coordinated and dynamic function can have a profound impact on injury and disease progression. The medicinal activity of MSCs is, thus, dependent on aspects of the management of the tissue and the site of injury or disease with respect to the therapeutic capacity of either endogenous or exogenously supplied MSCs. This infers that MSCs are intrinsically curative and that their therapeutic effectiveness solely depends on the "when, where, and how" of their delivery or presentation at sites of injury, disease, or regeneration.

3. When

At the site of any tissue injury, large or small, there is an immediate trigger to the acute inflammatory response which serves to bathe the site with molecules and cells to protect against invasion by toxic molecules or foreign organisms. This acute inflammation serves to also condition the site for either regeneration, repair, or scarring. The presence of MSCs following this initial flushing of the injury site would inhibit the intrusion of immune interrogating cells and further protect the site from the disbursement of agents that could be toxic to resident tissue cells. The activated MSCs function to inhibit connective tissue cells from pumping out massive amounts of collagen and other components that function as both the soil and the bed for scar. Thus, early in the injury response, sufficient numbers of MSCs could naturally serve to protect the injury field from degenerate events and allow regenerative repairs to be initiated. In this regard, in an aging individual with decreased numbers of MSCs, scarring would be more prominent.

Given the above logic, the "when" to deliver MSCs is after the major acute inflammation has died down, relatively early after the injury event. This could be at 48 hours after an acute myocardial infarct or by day 7 following a stroke as observed in preclinical animal models [14–19]. If the injury or disease state is chronic, multiple presentations of MSCs, say twice per week for 4 weeks (the Osiris Therapeutics, Inc., protocol for Crohn's Disease), anticipate multiple events and an extended duration of MSC exposure. In extreme cases such a heavily scarred tissue such as observed in patients with COPD or chronic asthma [21], again multiple exposures suitably spaced from one another should be required.

The issue of scarred tissue is quite complex and the age and health status of the subject are critical. Scar is a living tissue composed of massive ECM and its maintenance cells. The assumption is that scar, say in the lung, turns over. If MSCs do indeed function, either inhibiting the formation of scar or inhibiting the entrance or development of scar forming cells, then MSCs must reside at sites of scar for a considerable length of time or appear at critical intervals to inhibit scar formation or expansion while providing a microenvironment for the afflicted tissue to regenerate itself. In an animal model of asthma, multiple exposures to MSCs are required to enhance scar turnover and its eventual elimination [21].

4. Circulating or Mobilizing MSCs

The best data available indicates that MSCs do not circulate [22, 23]. Indeed, when MSCs were infused into the venous system of one arm only, a few MSCs could be detected right after infusion in the blood of the other arm, but none thereafter [22]. It is important to understand that if 100 million MSCs are slowly infused into the blood stream of an adult (even if all of these MSCs circulated which is improbable), the number of circulating blood cells is in such vast excess that it would almost be impossible to detect even one MSC by cell-sorting or by colony formation (MSC adhesion to culture dishes in optimal plating medium) [24].

This also begs the question as to when and if MSCs can be mobilized to sites of injury. The entire concept of "mobilization" stems from a misconception and faulty word-usage in hematology. It is commonly accepted to call the action of the drugs G- or GM-CSF as "mobilizing" because huge amounts of hematopoietic progenitors can be detected in peripheral blood samples [25, 26]. These drugs cause massive cell proliferation in bone marrow and the progeny becomes so densely packed that they push out through the sinusoids into the blood stream. This is a cell crowding event not cell-specific mobilization. Likewise, if rodents are grown in chambers of low oxygen, MSCs can be found in circulating blood consistent with an increase in blood levels of HIF-1α [27]. I believe the circulating MSCs are present because of numerous blood vessel breaks and the release of pericytes from their basal lamina anchorage not because of HIF-1α causes the mobilization of the cells.

The recent report that no circulating MSCs could be detected in various patients with chronic diseases but could be detected in patients with multiple fresh fractures does not disprove the concept that MSCs can circulate and can be mobilized [24]. The blood sample of the chronic disease patients contained no MSCs because the initiation of the chronic condition had long since passed and the micro "injury" to sustain a chronic condition is not known and difficult to time. Moreover, the sensitivity of a cell-sort or colony plating scheme is too low to detect MSCs if, indeed, they were mobilized and circulating. Again, for emphasis, the pericyte is released from its tether in the basal lamina at injury or inflammation to become an MSC that is both mobile and it can be swept into the blood stream. More basic information is required to understand these events *in situ* before we discard the notion that MSCs can be mobilized or that they circulate. The data involving SDF-1 (discussed below) could be used to argue that MSCs are motile and dock in specific regions of the vascular tree.

5. Where

MSCs function at sites of blood vessel damage or inflammation. That is where they need to be delivered. This can be accomplished by systemic delivery, but it is clear that these exogenous MSCs are fragile and can be eliminated almost immediately upon entering the blood stream [28]. Likewise, they can irreversibly lodge in the lung and liver [29] and, thus, never reach the tissue target. Therefore, where exogenous MSCs are introduced in the body can have a profound influence on their capacity to reach sites of recent or current injury or inflammation. This issue of "where" to infuse MSCs has been exquisitely documented by Lin et al. who introduced MSCs into mice via a carotid cutdown using a stiff catheter into the aortic arch and, thus, into the left ventricle and descending aorta whose blood flow bypasses the lung and liver for at least one full body passage [30]. These experiments were done in a mouse in which one leg was irradiated causing a marrow injury 4 hours prior to luciferase-labeled MSC infusion. The standard tail vein infusion uses one million MSCs while left ventricle infusion could deliver 10 times less yet document that the labeled MSCs did indeed dock in the injured leg marrow.

Direct injections of MSCs into synovial joints, spinal disc, or intramuscular are also being used clinically with apparent success. The most detailed study has involved a cork-screw catheter into an infarcted heart [31]. Penn and colleagues have shown that the infarcted rodent hearts released SDF-1 and that if exogenous MSCs are delivered [32, 33] within 48 hours after injury the MSCs will dock in this tissue and protect the heart from the subsequent damaging events. Importantly, if MSCs are introduced systemically on day 7, the SDF-1 is no longer being secreted and MSCs will not dock. Moreover, by using a plasmid for SDF-1 and delivering it to damaged heart, the SDF-1 subsequently produced will serve as a powerful chemoattractant for MSCs, presumably from marrow and other depots, to attract them to the injured tissue and to assist in both the protection and the recovery of the heart tissue [34, 35]. The sustained secretion of SDF-1 also holds promise for treating patients with chronic heart issues and is part of a current clinical trial (http://www.juventasinc.com/index.html).

Last, although systemic and direct injections of MSCs into afflicted tissue are in use, the introduction of MSCs into the peritoneal cavity has never been properly evaluated, especially for Crohn's disease, inflammatory bowel disease, or ailments of the abdominal region. Since the lymph tree in this cavity is so prominent, it is tempting to propose that MSCs might be highly effective if introduced into this tree. Likewise, would this tree be a useful port for systemic introduction of exogenous MSCs?

It must be emphasized that there is no quantitative information that elaborates the number of "docked" MSCs as related to a specific therapeutic outcome. The initial intravenous doses of MSCs are extraordinarily large in both rodent-disease models and in clinical trials where 1–5 million MSCs/kg are the standard doses. Moreover, although docking strategies have been employed, the efficiency of docking and the potency of MSCs are difficult to quantitate and almost impossible to relate to the composite therapeutic outcomes. As inferred above, if MSCs must dock in the damaged heart tissue and in the servicing lymph tree, the question of efficiency of docking and potency of MSCs becomes even more difficult.

6. How

Although clinical trials are now in play in which MSCs are mostly delivered intravenously, intramuscularly, and into the synovial joints, there are other routes of administration that are being explored. The cork-screw catheter was used to increase the needle path (creating an increase in focal injury) and to maximize the retention of MSCs in the heart and thus delivers MSCs into afflicted cardiac tissue where the MSCs not only dock in and on this newly injured tissue, but also spill out into the circulation [36, 37]. It may be that this spillage allows the MSCs to dock in the lymph system that services the heart where they may affect the local immune system (my speculation).

A very unusual, but potentially important delivery route has been published indicating that the upper sinus might be a perfect routing to the brain. Currently, intrathecal administration of MSCs is being used for patients with MS or ALS. Cells or drugs like insulin delivered to the upper sinuses are captured by a liquid stream that flows from around sensory axons of olfactory nerves up into the extracellular fluid that courses through in the brain from front to back [38, 39]. This may also be a more logical pathway for patients suffering from Parkinson's disease or MS to receive therapeutic cells including MSCs as has been published in rodents [40].

The therapeutic effect achieved by MSCs is by producing a spectrum of bioactive molecules that affect the injury site by both trophic and immunomodulatory mechanisms. The question arises as to whether, by priorly exposing MSCs to specific agents in culture, the paracrine activities could be optimized for a specific therapeutic outcome. For example, pretreatment of MSCs with IFN-γ protects [41] against graft-versus-host-disease (GVHD). Importantly, MSCs (unpretreated) mount an immunomodulatory assault on GVHD and two MSC products have been approved for use in children with steroid-refractory GVHD with substantially positive outcomes. Will IFN-γ pretreated MSCs eliminate all GVHD? This is doubtful given the complexities involved. However, if such pretreatment eliminated a sizeable proportion of GVHD upon bone marrow transplantation, this could save many lives and decrease the huge hospital costs.

Last, since cultured, exogenous MSCs are delicate and susceptible to damage upon entering the blood stream [28] or by direct injection into tissues; the encapsulation of MSCs may be a preferred route of administration with their subsequent slow release. For example, a small private company in Italy called Lipogems (for whom I currently consult) has an apparatus for treating lipoaspirate and generating 500 micron aggregates of adipocytes with MSCs trapped inside [42]. These aggregates when introduced into culture do not plate out, but MSCs can be observed to crawl out onto the plate after 4–7 days. Such autologous MSCs would appear at sites of injury after the acute inflammatory phase of their introduction and could be then highly effective. Clinical use for fecal incontinence, osteoarthritis, muscle injury, and so forth has been reported to be highly effective. Proper double-blind, placebo control clinical trials should be quite interesting for this MSC slow release and protective technology.

7. Who Makes the Therapeutic Molecules?

Because MSCs function medicinally at sites of injury, it is assumed that they produce a spectrum of therapeutically active molecules. But, do they? An ingenious experiment has been performed by Adonis Hijaz, MD, and his colleagues [43] (Hijaz et al., personal communication). A urinary incontinence model is generated in rodents by placing a balloon in the animals' vagina. The urethra is injured causing leakage of urine that can be quantitatively accessed by leak-point pressure. If human MSCs labeled with a fluorescent dye are introduced into the urethra, the animal is back to normal by day 4. If, on day 1, the animal is sacrificed and the urethra sectioned, laser capture microscopy can isolate tissue containing the fluorescently tagged MSCs and tissue situated next to the labeled hMSCs. In a separate injured animal, the injured tissue that has never been exposed to hMSCs can be isolated by laser capture techniques. RNA chips using purified RNA from these laser captured specimens indicate that the hMSCs are making many different molecules compared to what they originally made on the Petri dish from which they were expanded and isolated. More interesting is the fact that injured tissue situated next to the hMSC is making over 90 different molecules compared to the injured tissue that had never been exposed to the hMSC. By using both rodent specific RNA chips and human specific RNA chips, the question can be asked at that one time point: who is making the therapeutically relevant molecule the rodent host tissue or the hMSC? A more detailed temporal analysis will be needed to not only answer this question, but establish the dynamic interaction between the hMSC and the injured tissue. Having stated this question related to the source of secretion of the therapeutic molecules, the introduction of MSCs acts to inhibit scarring and stimulate *de novo* regeneration [15, 16].

The reason for reviewing the above is to emphasize the emerging theme that MSCs appear to be assisting the host tissue to maximize its *intrinsic* regenerative capacity. The local management of the immune cells and the tissue specific progenitors appears to be accomplished by very few, locally situated, and short-lived MSCs. This innate regenerative capacity of almost every host tissue has never been properly managed except, perhaps, in orthopedics where the vigorous regenerative and repair capacity of bone (maybe through local MSCs) is managed by orthopedic physician interface.

The Medicine of Tomorrow may be the management of MSCs to optimize the body's very powerful and ever-changing intrinsic regenerative potential.

Conflict of Interests

The author declares that Case Western Reserve University receives royalties from Osiris Therapeutics some of which are shared with him to cover his formation of Osiris Therapeutics and patents transferred out of the university.

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06

ARTICLE

Alopecia and platelet-derived therapies

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Original Article

Stem Cell INVESTIGATION

Alopecia and platelet-derived therapies

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Background: Platelet rich plasma (PRP) injections have emerged as a promising regenerative therapy for androgenetic alopecia (AGA). To date, injections of both autologous native and activated PRP have been administered to hair loss patients, and positive results have been observed. However, little to no work has yet to be seen wherein PRP treatments are combined with hair restoration surgeries. Furthermore, the PRP activation protocol in the hair restoration setting employs compounds with potentially deleterious side effects, namely thrombin or calcium gluconate. Therefore, the objectives of this work are to evaluate the effectiveness of platelet and platelet-derived products as augmented graft therapies in hair restoration surgeries and to compare the follicular regeneration rate of follicles transplanted in the presence of platelet lysate (PL) versus activated PRP (AA–PRP).

Methods: PL was administered to the frontal scalp of three male AGA patients. Three treatment zones measuring 4 cm^{-2} were mapped in the midline scalp region of each patient and equal number of follicular grafts were placed in each box along with PL, AA–PRP, or normal saline. The transplanted follicular grafts of a fourth patient were placed solely with PL. Hair checks in which the surface area of hair coverage was quantified were performed at follow-up appointments ranging from 3.5 to 7 months post-surgery. In these appointments, the number of follicular units with hairs measuring 50 mm or more were counted to determine the percentage of graft hair regeneration. Growth factor (GF) concentrations [vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF-β1), PDGF-BB, IGF-1] in PL and AA–PRP were also measured for an independent subject set.

Results: Follicular regeneration in transplanted grafts was found to be superior for those placed with PL rather than AA–PRP or saline at all follow-up dates. Specifically, at 3.5 months post-op, 89%±9%, 74%±7%, and 57%±10% of follicular units had regenerated hair in the PL, AA–PRP, and saline treatment zones, respectively. At 4 months post-op, 99%, 75%, and 71% of follicle regeneration had occurred in the PL, AA–PRP, and saline treatment areas, respectively. Impressively, when PL was injected alone, the patient experienced a 50% increase in follicular unit density and a 122% increase in hair density 7 months post-injection. When GF concentrations were measured, PL generated from a 30-min sonication of PRP was found to have significantly higher levels of VEGF, PDGF-BB, and TGF-β1 than AA–PRP.

Conclusions: PRP remains a promising hair loss therapy and should be evaluated further for use not only as an independent therapeutic tool, but also as a treatment to augment surgical procedures. PL in particular affords an effective and efficacious therapeutic product given that the lysate may be obtained by mechanical rather than chemical means. Ultrasonic waves provide sufficient energy to rupture platelet cell walls, and centrifugation may be used to separate the lysate from cell fragments prior to delivery.

Keywords: Androgenetic alopecia (AGA); platelet rich plasma (PRP); concentrations

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Introduction

Hair loss disorders, such as androgenetic alopecia (AGA), display relatively high rates of occurrence in both men and women. Fifty percent of white men will experience some degree of AGA, also known as male-pattern baldness or MPHL, by age 50 (1), and nearly 50% of women will experience AGA (female-pattern hair loss, FPHL) over the course of her lifetime (2). Given the prevalence of hair loss in these populations, targeted therapies that reduce the appearance of thinning by delaying, arresting, or reversing the underlying pathology are highly desirable. However, the current selection of pharmacotherapies with US Food and Drug Administration (FDA) approval are limited to oral finasteride, a selective 5-α-reductase inhibitor, and 2% or 5% topical minoxidil solutions or foams (3,4), neither of which enable complete hair regrowth, even with persistent use. Moreover, the patient will inevitably face relapse in hair loss if and when the treatment is halted, and undesirable side effects may result from these medications, some of which, such as finasteride-induced impotence, may persist for many years (5).

Alternative cell-based therapies for the treatment of AGA have emerged, of which, platelet rich plasma (PRP) injections appear particularly effective. Although the precise degree to which PRP, improve hair density is difficult to extract from individual studies given the relatively small sample sizes and lack of standardization in follow-up periods, test factors measured, and instruments used to quantify hair count and/or quality, a recent meta-analysis identified PRP as a promising treatment for AGA (6). In practice, autologous PRP (A–PRP) may be applied directly or activated (AA–PRP) prior to delivery. When A–PRP has been injected in its native stage, hair density improvements have ranged from 13% to 31% over baseline measurements 3 months following the initial treatment (7,8). Treatment with AA–PRP injections has shown similar hair density improvements 12 to 14 weeks after therapy was begun, with hair densities ranging from 18% to 29% above baseline (9-11). However, hair density measurements made 6 months after the start of AA–PRP treatment have shown up to a 56% improvement over baseline values, although the degree of improvement seems to be influenced by the system used to generate the PRP from whole blood (7).

Since PRP therapies are still in their infancy, and have not been shown to arrest or reverse the hair loss process, many AGA patients turn to surgical intervention to conceal ongoing thinning (12). Within the hair restoration arena, the minimal depth follicular unit isolation technique, particularly when conducted with sharp punches, has proven to be cosmetically effective and affords markedly low transection rates in skilled hands (13). The "minimal depth" ranges from 1.8 to 3.0 mm below the scalp surface, which places the extraction punch in the deeper dermis or superficial adipose tissue at full stop. Since the average hair follicle extends 4.2 mm (range, 4–5.1 mm) into the scalp (14), the minimal depth extraction approach allows valuable cell populations to remain intact following follicular extraction, namely, epidermal stem cells distal to the sebaceous gland in the bulge region and mesenchymal stem cells in the dermal papilla (15).

In addition to the minimally invasive surgical procedure that conserves vital stem cell populations within the donor region of the scalp, drug therapies that promote wound healing and accelerate regeneration of transplanted hair follicles may also be applied. Products such as hyaluronic acid, extracellular matrix proteins, or PRP may be used either singularly or in an assortment of combinations (16). Uebel *et al.*, for example, have immersed extracted grafts in A–PRP then activated the PRP immediately prior to graft implantation (17). In this work, grafts implanted with AA–PRP displayed significantly higher rates of survival over controls. To date, A–PRP activation, in the context of hair restoration surgeries, has been accomplished via thrombin, calcium chloride, or calcium gluconate solutions as a means to deliver GFs that are either secreted by the platelets or presented on the activated platelet surface (18). However, since each of these products constitute a unique drug therapy with accompanying unwanted or potentially hazardous side effects, an alternative process by which platelet contents may be isolated is warranted for efficacious clinical use. Platelet lysis and accumulation of the released lysate (PL) is one solution, which is accomplished herein via sonication. In this work, the survival rates of hair follicles treated with PL, AA–PRP, or normal saline following transplantation are compared, and the relative growth factor (GF) concentrations for PL and AA–PRP are evaluated.

Methods

Patient population

This study enrolled 3 male patients aged 27–55 years who displayed MPHL in Stage 6 as determined by the Norwood-Hamilton classification scale. For each patient, three sets of 2 cm × 2 cm (or 4 cm^2 area) boxes were marked

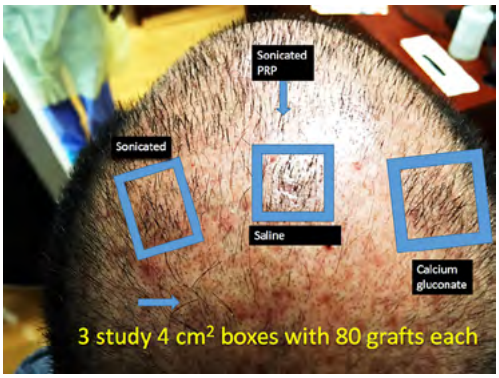


Figure 1 Location of the PL, AA–PRP, and saline treatment zones for Subject A. Eighty grafts were placed in each box, and hair checks were conducted 14 weeks post-surgery. PL, platelet lysate; AA–PRP, autologous activated platelet rich plasma.

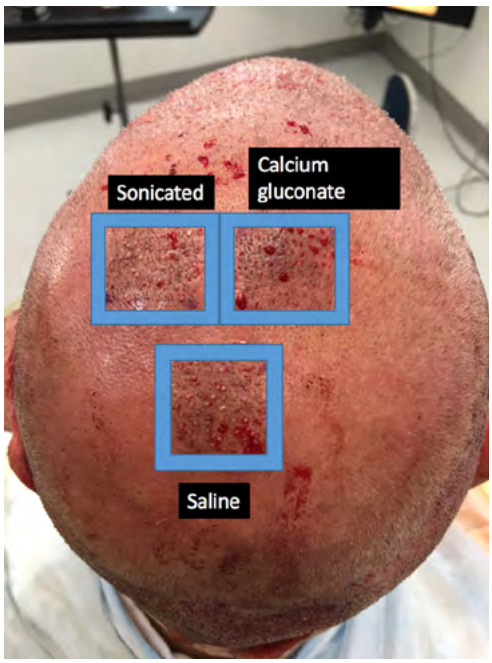


Figure 2 Location of the PL, AA-PRP, and saline treatment zones for Subject B. Eighty grafts were placed in each box, and hair checks were conducted 18 weeks post-surgery. PL, platelet lysate; AA–PRP, autologous activated platelet rich plasma.

in three separate regions of the transplant zone. Follicular unit grafts in conjunction with 1.0 mL PL, 1.0 mL calcium gluconate-activated PRP (AA–PRP), or 1.0 mL normal saline were then placed in each box. The locations of the respective treatment, positive control, and negative control

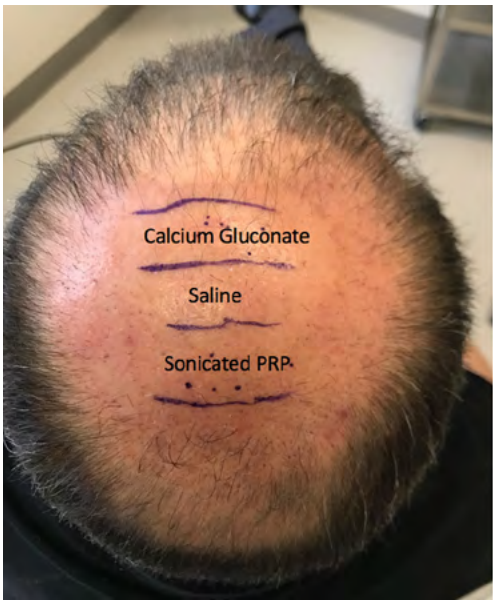


Figure 3 Location of the PL, AA-PRP, and saline treatment zones for Subject C. Forty grafts were placed in each box, and hair checks were conducted 14 weeks post-surgery. PL, platelet lysate; AA–PRP, autologous activated platelet rich plasma.

boxes for each patient are outlined in *Figures 1–3*. Note, however, that the number of hairs within each follicular unit were controlled only in subject C. Only the absolute number of grafts, not the absolute number of hairs was held constant among the three regions in subjects A and B. The frontal scalp was treated with 0.1 mL cm^{-2} PL in all patients. A second group of 5 subjects (3 male and 2 female aged 20–60 years) with no apparent hair loss was selected solely for analyzing GF concentrations in PL and AA–PRP.

A–PRP preparation, activation, and lysis

A–PRP was prepared using one of two systems. In the first, Regen Blood Cell Therapy (BCT) tubes were used to prepare A–PRP (15 and 5 mL per BCT tube) from whole blood (24 mL) taken from a peripheral vein using sodium citrate as an anticoagulant. The top 2 mL of A–PRP from each tube was then discarded, giving 9 mL of A–PRP with a 5-fold increase in platelet concentration over whole blood. The Regen system A–PRP was activated through the addition of 10% (v/v) calcium gluconate, and the resulting AA–PRP was immediately injected at a depth of 3 mm into the appropriate treatment zone through a 1 mL Luer lock

Figure 4 The counting incision device (Cole instruments) used to determine the number of follicles with regenerated hair.

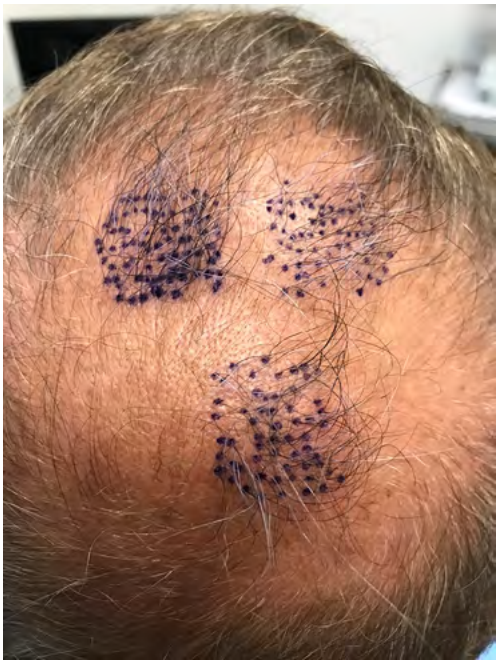


Figure 5 A photograph illustrating the graft recording hair check process. Counted follicular units with hair(s) measuring at least 50 mm are marked in violet for the treatment regions in Subject B.

syringe equipped with a 25-gauge needle.

The Arthrex Angel system was also used to prepare A–PRP (3 mL) from 120 mL of whole blood with the instrument hematocrit level set to 3%. The A-PRP was then combined with 5 mL of platelet poor plasma to produce 8 mL of A–PRP with a 5-fold increase in platelet concentration over whole blood. A–PRP collected with the Arthrex Angel system was subjected to 30 s on/30 s off sonication cycles using a Bioruptor Plus sonication device (Diagenode) equipped with six falcon 15 mL tube holders, each filled with 1.33 mL of A–PRP and maintained at 4 ℃ over the entire sonication period by an accompanying water cooler (115 V minichiller, Diagenode). Total sonication time was set to 30 min (i.e., 60 on/off cycles). Upon completion of the final cycle, the lysed platelet samples were centrifuged for 10 min at 1,967 ×g, and the supernatant (PL) was recovered and delivered at a depth of 3 mm into the appropriate treatment zone through a 1 mL Luer lock syringe equipped with a 25-gauge needle.

Assessment of hair growth parameters

For each patient, individual follicular units were counted in each box using a counting incision device (Cole Instruments) loaded with a gentian marker to prevent duplicating the count for any follicular unit and to record a precise number (see *Figures 4,5*). The number of growing follicular units measuring at least 50 mm were recorded for each of the three regions of interest, that is, the PL, AA–PRP, and saline-treated grafts.

GF quantification

AA–PRP and PL samples intended for GF concentration testing were prepared as described above. For PL samples, the total sonication time was set to 0 (no treatment), 15 min (i.e., 30 on/off cycles), or 30 min (i.e., 60 on/off cycles). All samples were centrifuged for 10 min at 1,967 ×g, and the recovered supernatant was stored at 4 ℃ prior to testing. The concentrations of four GFs [PDGF-BB, IGF-1, transforming growth factor beta 1 (TGF-β1), and vascular endothelial growth factor (VEGF)] were measured using commercially-sourced enzyme-linked immunosorbent assay (ELISA) kits (*Table 1*) in accordance with standard, previously reported protocol (14). Briefly, standards and samples were added to a 96-well microplate pre-coated with an antibody against the target GF. An enzyme-linked polyclonal antibody, specific for the target GF was added in excess and the unbound antibody was rinsed away. A

substrate solution was then added and color developed in proportion to the quantity of bound GF. After the color development was stopped, the absorbance was measured at 450 nm using a µQuant microplate reader (Bio-Tek). GF concentrations were determined from standard curves with the aid of GraphPad Prism curve-fitting software. For IGF-1 and TGF-β1, the data was linearized by plotting the log(concentration) versus the log(OD450), and a best fit was found via linear regression analysis. For all others, a nonlinear four-parameter logistic curve fit was performed. For Regen-derived GF measurements, n=5. GF measurements for the Arthrex Angel system contained only 2 of the above 5 subjects (n=2).

Statistical analysis

Platelet GF concentrations are expressed as mean ± standard error (n=5 or n=2). Unpaired *t*-tests (α =0.05) were used to determine differences in mean among the baseline GF concentrations in A–PRP derived from the Regen and Arthrex systems. One-way Analysis of Variance (ANOVA) was used to determine statistical significance between data sets (α =0.05). For data sets with significant differences in means, the Tukey test was applied to identify the source(s) of variation

Table 1 Commercially-sourced ELISA kits used to quantify platelet growth factors

Target protein	ELISA assay kit specifications
PDGF-BB	Cat # EHPDGFB, Thermo Scientific
VEGF	Cat # KGH011, Novex Life Technologies
IGF-1	Cat # DG100, R&D Systems
TGF-β1	Cat # KAC1688, Invitrogen Life Technologies

ELISA, enzyme-linked immunosorbent assay; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor beta 1.

Results

The number of visible follicular units within the PL, AA–PRP, and control boxes at follow-up, in addition to the number of follicular units placed within each box during the surgical procedure, are provided in *Table 2* for the three primary participants of this study. Transplanted follicular unit growth was highest in those regions treated with PL, where 89%±9% and 99% of follicular units were visible at 14 and 18 weeks, respectively. Although the AA–PRP region appeared more successful than the control at 14 weeks with 74%±7% versus 57%±10% of transplanted grafts visible, there was little disparity between these two groups at 18 weeks (75% versus 72% for AA–PRP and saline treatment zones, respectively).

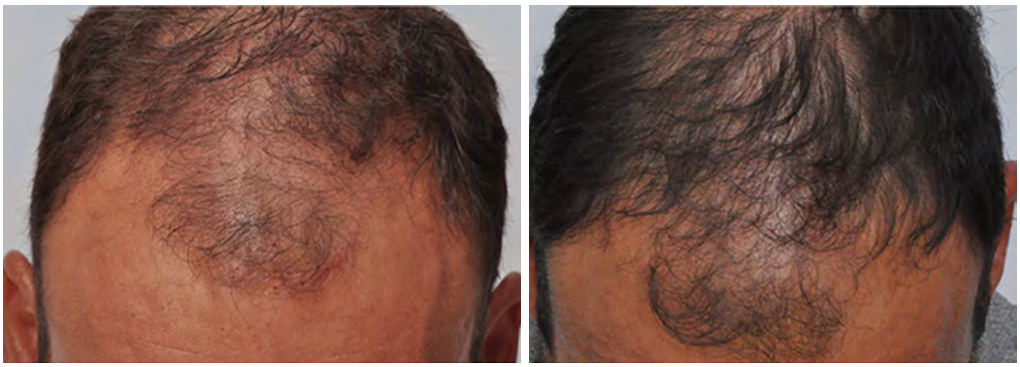
For comparative purposes, the entire scalp of a single subject (Subject D, 44, Norwood Five) was treated exclusively with PL (10 mL). Four tattoos were drawn on the mid-scalp, and hair checks were performed on these regions at 28 weeks post-treatment. At this time, the patient demonstrated an increase in visible follicular unit density and hair density from starting values of 60 FU cm^{-2}, and 90 hair cm^{-2} to 90 FU cm^{-2}, and 200 hair cm^{-2}. Before and after photos for this patient are shown in *Figure 6*. As a control, 18 grafts were placed into a scar and treated only with saline. At 16 weeks, only 10 of these grafts were visible.

Concentrations of IGF-1, TGF-β1, PDGF-BB, and VEGF for intact and lysed Arthrex-derived PRP and native and activated Regen-derived PRP are provided in *Table 3*. No significant difference was observed between native PRP collected via Regen BCT or the Arthrex Angel system for any of the GFs evaluated in this study. Overall, the highest concentrations were obtained from Arthrex PRP samples that underwent a 30 min sonication. One notable exception was the IGF-1 value, as no significant difference in means was found for this GF (P=0.98). For the remaining three GFs

Table 2 Follicular units placed and counted in the PL, AA–PRP, and control regions at follow-up for the primary subjects

Subject	Follicular units placed per 4 cm^2	Follow-up (weeks)	Follicular units (no.)		
			PL	AA–PRP	Control
A	80	14	64	54	53
B	80	18	79	60	57
C	40	14	39	32	19

PL, platelet lysate; AA–PRP, autologous activated platelet rich plasma.



Before Six months after sonicated PRP

Figure 6 Before and after photos of Subject D. Hair check in the after photo was performed 28 weeks post-injection.

Table 3 PL, PRP, and AA–PRP growth factor concentrations

Growth factor	Regen BCT		Arthrex angel system		
	PRP	AA–PRP	PRP	PL–short†	PL–long‡
IGF-1 (ng mL^{-1})	130±20	140±20	150±40	140±40	150±40
TGF-β1 (ng mL^{-1})	11±2	15±3	12±1	50±8	93±30
PDGF-BB	1.2±0.3	4±2	1.1±0.6	19±0	24±2
VEGF (pg mL^{-1})	61±20	210±40	61±20	920±30	1630±470

†, PL generated from 15 min total sonication time; ‡, PL generated from 30 min total sonication time. PL, platelet lysate; AA–PRP, autologous activated platelet rich plasma; BCT, Blood Cell Therapy; TGF-β1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor.

(i.e., TGF-β1, VEGF, and PDGF-BB), a 30-min sonication produced significantly higher concentrations than did activation with calcium gluconate, which was not significantly different from native Regen PRP with respect to any GF.

Discussion

The results of this work show that transplanted grafts resume growth faster in the presence of PL than treatment with AA–PRP or saline. However, for Subject A, it was noted that the quality of grafts in the Regen-derived AA–PRP region was superior to those in the Arthrex-derived PL region upon initial placement and in the follow-up evaluation at 14 weeks. Even though the coverage was aesthetically better in the AA–PRP box, the number of grafts growing was higher in the PL box, a phenomenon likely attributable to the grafts in the Regen box coincidentally containing a greater number of hairs per follicular unit than those placed in the PL box. Nevertheless, the faster rate of regrowth for grafts placed in the presence of PL cannot be disputed. Indeed, the singular, beneficial influence of PL in hair regeneration is best illustrated in Subject D, as this patient was treated exclusively with PL over his entire recipient region; no graft transplantations were performed. As previously noted, the frontal regions of Subjects A, B, and C, were treated with PL, and since vascular flow from the frontal scalp to adjacent regions of the scalp cannot be blocked, the potential for follicular regeneration within the AA–PRP or saline boxes to be positively influenced by PL injected in the frontal scalp could not be avoided entirely. Importantly, the locations of the three test boxes in the recipient region of the scalp were shuffled in an effort to account for any contamination of the results.

A plausible hypothesis for the superior follicular regeneration rate of PL-treated grafts over AA–PRP-treated grafts may be drawn based on the relative GF concentrations in PL and AA–PRP, respectively. With the previously noted exception of IGF-1, which was statistically

equivalent for all test groups, PL contained significantly higher GF concentrations than AA–PRP. As suggested by their names, GFs promote cell growth through several pathways. VEGF, for example, promotes angiogenesis, which establishes a conduit for nutrient delivery and waste removal (19), and TGF-β1 prevents basal keratinocytes from reaching terminal differentiation which ensures the proliferative potential of this cell population is retained (20). When dermal papilla cells are cultured in the presence of activated PRP, higher concentrations of molecules linked to hair growth are measured with respect to controls. Specifically, higher levels of β-catenin, the protein in the Wnt pathway that is responsible for stimulating hair follicle development (21,22), have been observed (23). Thus, it is reasonable to expect grafts placed with higher concentrations of platelet-derived GFs to regenerate hair follicles sooner than those placed with lower concentrations of the same molecules.

Given that PL and AA–PRP are derived from identical sources (i.e., platelet concentrated whole blood), one may question why PL contains markedly higher levels of most GFs. The answer likely stems from the mechanism by which platelets relinquish GFs in the respective processes. When platelets are activated by exposure to calcium gluconate, GFs are released from the α-granules and glycoprotein IIb/IIIa, a receptor able to bind fibrinogen, is translocated to the cell membrane (18). Binding of fibrinogen to glycoprotein receptors on adjacent platelets causes them to adhere to one another, and coagulation ensues. Ultimately, a platelet plug is formed, within which a high concentration of GFs is trapped. The rate at which these bioactive proteins can reach surrounding tissues to initiate their respective pathways becomes diffusion- and mesh size-limited. Small proteins should be released from the plug faster than large proteins which may require some degree of plug degradation before they are released. Alternatively, when platelets are lysed, the contents of α-particles are released without aggregation, as the cell walls are no longer intact. In terms of a therapy tool, the limiting factor for concentrating GFs becomes the percentage of platelets that are lysed in a given sonication session.

The relative GF concentrations measured in this study indicate that molecular size does influence recovery in a method dependent manner. IGF-1 (7.6 kDa), a protein that is substantially smaller than TGF-β1 (~25 kDa), PDGF-BB (~30 kDa) or VEGF (~38 kDa) (24-26), was present in statistically equivalent concentrations for all PRP treatments. The remaining GFs, which are very similar in size, exhibited a six- to eight-fold greater concentration in PL originating from a 30-min sonication period than in AA–PRP. Conceivably, these larger proteins were not readily expelled out of the platelet plug upon centrifugation owing to pores within the plug being too small.

The immediate and positive influence of a bolus GF delivery from PL on follicular regeneration has been discussed above; nevertheless, given the upward trend in follicular unit density and hair density when PL is administered as a stand-alone therapy, one may conclude (albeit with limited confidence, n=1, Subject D) that secondary factors are at play. We hypothesize that PL may afford activation of additional platelets and/or initiation of relevant hair stimulation pathways more effectively than AA–PRP since the platelet cell membranes are removed. In PL, molecules that participate in the clotting cascade (i.e., ADP and ATP from dense granules) or that are released to activate additional platelets (i.e., thromboxane A2 from α-granules) are free to diffuse into the surrounding tissue without being bound by platelets within the venous blood draw that were concentrated into the PL precursor, A–PRP. Since the precise cellular mechanisms encompassing the beneficial nature of PL in hair replacement surgeries has yet to be identified, the authors recommend that this field be more thoroughly examined given the promising, side effect-limited nature of the therapy.

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Footnote

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

Ethical Statement: Informed consent was obtained from all study patients.

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07

ARTICLE

Mechanisms involved in the therapeutic properties of mesenchymal stem cells

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Mini review

Mechanisms involved in the therapeutic properties of mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) have been described as being able to give rise to several quite different mesenchymal cell phenotypes. However, the ability to differentiate is not the only characteristic that makes these cells attractive for therapeutic purposes. The secretion of a broad range of bioactive molecules by MSCs, such as growth factors, cytokines and chemokines, constitutes their most biologically significant role under injury conditions. Understanding this intricate secretory activity as well as the properties of MSCs *in vivo* is central to harnessing their clinical potential. Herein, we identify some of the molecules involved in the paracrine effects of MSCs with a perspective that these cells intrinsically belong to a perivascular niche *in vivo*, and discuss how this knowledge could be advantageously used in clinical applications.

1. Introduction: mesenchymal stem cells

Adult mesenchymal stem cells (MSCs) can be defined as multipotent cells able to differentiate into various types of end-stage, specialized mesenchymal cells such as osteoblasts, chondrocytes, adipocytes, tenocytes and others [1]. Research involving MSCs can be traced back to the 1970s, when A.J. Friedenstein and colleagues described an adherent, non-hematopoietic cell type present in the bone marrow (BM) of different species that could form fibroblastic colonies *in vitro* (reviewed in [2]). These cells were termed fibroblastic colony-forming units (CFU-Fs), and the *in vitro* progeny of CFU-Fs were later found to have the ability to differentiate along osteogenic, adipogenic and chondrogenic pathways *in vitro* and when implanted *in vivo* (reviewed in [3] and [4]).

The introduction of a culture system for the study of hematopoietic stem cells (HSCs) by T.M. Dexter and colleagues [5] facilitated the elucidation of the cell hierarchy of the hematopoietic system. The notion that CFU-F can be found in the "stromal" adherent layer of Dexter-type cultures [6] led to the notion that this cell type is present in the BM stroma, and the term "stromal cell" became popular. The existence of a stromal system, with a stromal stem cells at the top of the hierarchy, was proposed by M. Owen [7]. At nearly the same time, based on an analogy with the hierarchical system described for HSCs, the existence of an MSC at the top of a hierarchy of non-hematopoietic cells in BM was proposed [1]. This proposed non-hematopoietic hierarchy would be able to give rise to cells characteristic of connective tissues, such as bone, cartilage, adipose, tendon, ligament and others, but not stromal components such as endothelial cells or macrophages [1]. Accordingly, plastic-adherent BM cell populations have been operationally defined as MSCs based on their ability to proliferate in culture and to differentiate when maintained under appropriate conditions *in vitro* or when implanted *in vivo* (reviewed in [8]).

The notion that BM contains MSCs has been and still is useful for the purpose of tissue engineering, which relies on the ability of these cells to differentiate into tissue-specific cell types when combined with biomaterials and given proper differentiation stimuli [9]. The same concept has also allowed the suggestion that MSCs are distributed throughout the body, as different research groups, using various organs as cell sources, have derived cells able to proliferate and differentiate into mesenchymal cell types (reviewed in [10]).

2. Conceptual problems regarding MSCs

Currently, there is much debate in the field as to how to refer to the plastic-adherent cells operationally defined as MSCs. The International Society for Cellular Therapy (ISCT) has proposed that human fibroblast-like, plastic-adherent cells be termed "multipotent

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mesenchymal stromal cells," regardless of the tissue from which they are isolated, and that the term "mesenchymal stem cell" be used only for cells that meet clearly stated stem cell criteria [11]. Subsequently, ISCT proposed minimal criteria to define multipotent mesenchymal stromal cells: the cells should be adherent to plastic; positive for CD73, CD90 and CD 105 and negative for CD11b or CD14, CD19 or CD79α, CD34, CD45 and HLA-DR; and differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [12]. However, these criteria will require some adjustments as research describing MSCs obtained from tissues other than BM advances. Short-term cultured MSCs from human adipose tissue, for example, express CD34 [13,14]. It is likely that suggestions for changes in the nomenclature concerning MSCs will also emerge with the advancement of knowledge regarding MSCs *in vivo*. In this review, we will take advantage of the acronym overlap between "mesenchymal stem cells" and "multipotent stromal cells" and refer to this particular cell population as "MSCs."

Another interesting point regarding MSCs is the question as to whether or not cultured MSCs represent homogeneous or heterogeneous populations. A study published in 1999 showed that MSCs obtained by adherence to plastic and cultured in pre-selected lots of fetal bovine serum [15] were nearly homogeneously positive or negative for 48 molecules, which suggests that MSC cultures represent homogeneous populations [16]. Clonal analyses of human MSCs have shown that, at a given moment during cell culture, there are clones able to differentiate into three, two or only one mature cell type assayed, and that the frequency of tripotent clones decreased as the cells were expanded in culture [17]. When murine MSCs were plated at a single cell per well, the frequency of colony-forming clones was found to be nearly 50%, and when some of these clones were expanded and analyzed, they showed phenotypic characteristics similar to those of the parental populations [18]. These results indicate that cultured MSCs are heterogeneous, stem cell-containing populations. Furthermore, since mature cells have been shown to dedifferentiate in culture and express markers considered to be characteristic of MSCs [19], it is possible that mature cell types also contribute to the establishment of MSC cultures and, by extension, to culture heterogeneity. For example, when CD34+CD31+ (endothelial) cells are sorted out of primary cultures derived from the stromal/vascular fraction of human adipose tissue and further cultured under MSC conditions, they lose expression of these markers and display a surface profile comparable to that of MSCs (Fig. 1).

As mentioned above, much of what is known about MSCs derives from experiments involving cultured cells, which makes the task of transposing the *in vitro* results to an *in vivo* context difficult. So far, MSCs have not been unequivocally identified *in vivo*, partly because there is no established consensus on what markers can reliably identify MSCs *in situ*. The use of markers of cultured MSCs to search for these SCs *in vivo* is hampered by the fact that expression of these markers may be spuriously determined by the culture conditions rather than characteristic of MSCs *in situ*. In spite of this drawback, this approach has allowed the suggestion that MSCs are associated with blood vessels *in vivo* [20–23]. And, indeed, earlier and recent reports suggest that pericytes, cells that are located on the abluminal side of blood vessels in close contact with endothelial cells, show great similarity to MSCs *in vitro* [21,24–27] and may behave as tissue-specific SCs *in vivo* [28–36]. The finding that the frequency of fibroblastic colonies observed in CFU-F assays strongly correlates with vascular density is a natural consequence of the association of MSCs with blood vessels [37]. Further support for the view that the perivascular niche is a common stem cell microenvironment for resident MSC-like populations within different vascularized tissues comes from the demonstration that CD146+ cells isolated from the retina (putative pericytes, as described below) display properties similar to those of MSCs isolated from multiple adult and fetal tissues, such as the capacity for differentiation toward adipogenic, osteogenic and chondrogenic lineages ([25]; reviewed in [38]).

The term "pericyte" is used here for the sake of simplification, as many names can be used for these periendothelial cells depending on their anatomical location and whether or not they are embedded in the basement membrane that surrounds blood vessels (e.g., perisinusoidal cells in sinusoids, adventitial reticular cells in BM sinusoids, Ito or stellate cells in the liver, mesangial cells in kidney glomeruli, and so on). The perspective, in light of both physiological and injury circumstances, is that some pericytes are SCs in the tissues from which they originate and correspond to MSCs in connective tissues [10]. This proposition does not imply that pericytes/MSCs from all organs are equivalent; indeed, MSCs obtained from different tissue sources show some differences regarding differentiation potential [18,37] and gene expression profiles [25,39–41]. Differences in gene expression are observable between MSCs obtained from different fetal organs, which suggests that they are already determined in prenatal life (Fig. 2).

In spite of the existence of abundant evidence indicating that the best *in vivo* candidate for the role of MSC in connective tissues is the pericyte, definitive proof that this cell type can self-renew for a lifetime is still lacking. Recently, Sachetti et al. showed that clonal, CD146+ adventitial reticular cells (BM pericytes) are able to organize a hematopoietic environment when implanted in mice after *in vitro* expansion, and do so again when re-transplanted into secondary recipients [42]. However, this finding alone does not provide strong basis for the assumption that the cells used are representative of or behave as stem cells *in vivo*, as they were subjected to culture before implantation and, most importantly, no evidence of self-renewal *in vivo* was presented as the absolute

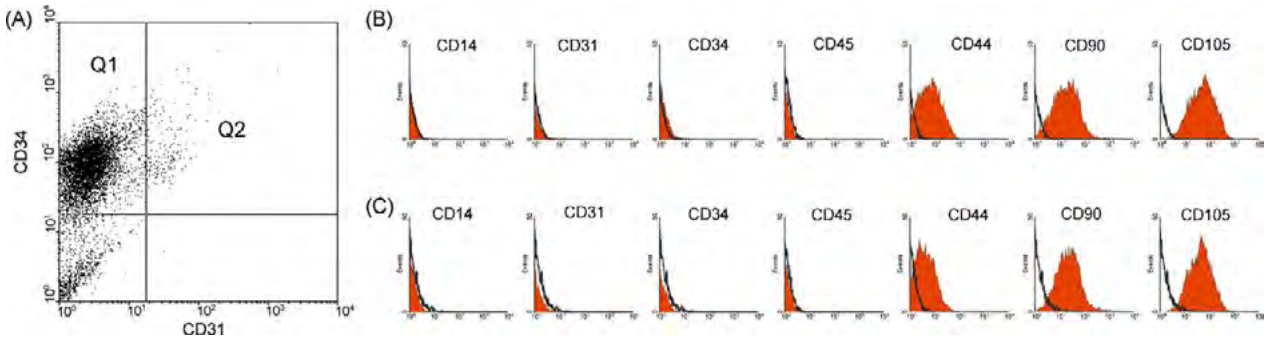

Fig. 1. Loss of expression of CD34 and CD31 by adipose tissue-derived cells. CD34+CD31− cells (Q1, panel A) and CD34+CD31+ cells (Q2, panel A) were sorted out of a 4-day-old primary culture of stromal/vascular cells from human adipose tissue and culture-expanded for an additional 3 and 2 passages, respectively. The surface marker profile exhibited by the progeny of CD34+CD31− cells (panel B) and CD34+CD31+ cells (panel C) is comparable to that commonly attributed to MSCs.

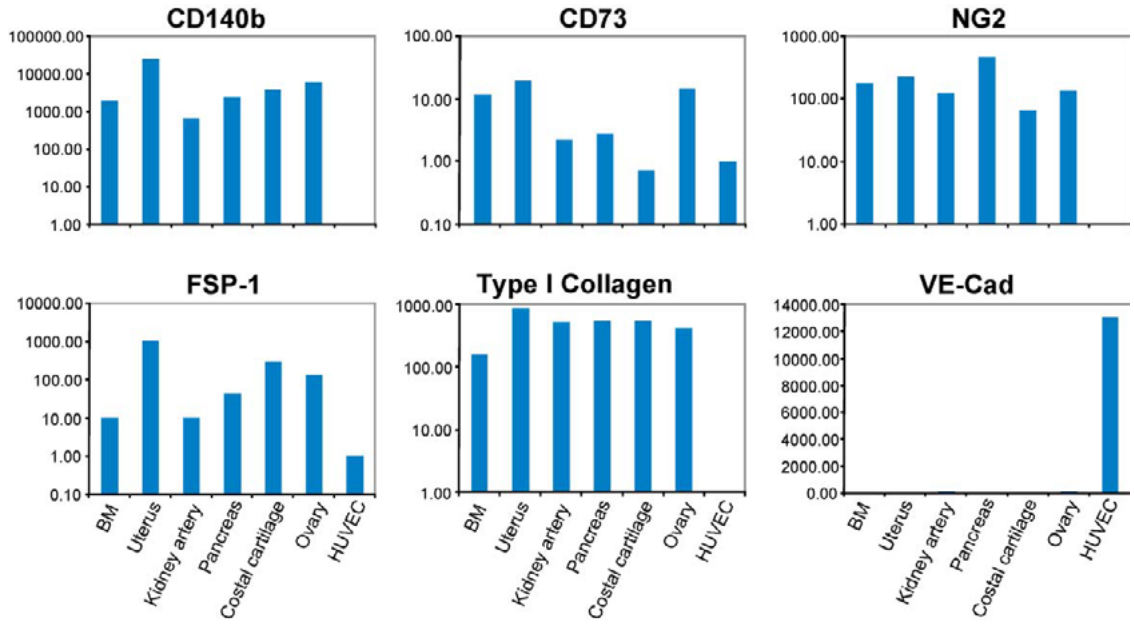

Fig. 2. Comparison between the expression levels of selected genes in MSCs derived from adult bone marrow and various fetal tissues. Transcript levels of CD140b, CD73, nerve/glial antigen 2 (NG2), fibroblast-specific protein 1 (FSP-1), collagen type I and vascular endothelial (VE)-cadherin were evaluated by real time PCR. Cultured MSCs from bone marrow and five fetal tissues including uterus, kidney artery, pancreas, costal cartilage and ovary were analyzed, and human umbilical vein endothelial cells (HUVECs) were used as controls.

numbers of cells present in the recipients were not assessed. Demonstrating self-renewal of putative MSCs *in vivo* is technically difficult because the turnover rate of connective tissues is far lower than that of hematopoietic tissue [43].

3. Conceptual problems aside, MSCs are promising therapeutic tools

Recently, the concept of MSCs discussed above has been broadened to include the secretion of biologically active molecules that exert beneficial effects on other cells [44]. This shifts a paradigm centered on differentiation to a view in which MSCs can be therapeutic even if they do not engraft or differentiate into tissue-specific cells, which significantly increases the range of MSC therapeutic applications. MSC paracrine effects can be divided into trophic ("nurturing"), immunomodulatory, anti-scarring and chemoattractant. The trophic effects of MSCs can be further subdivided into anti-apoptotic, supportive (stimulation of mitosis, proliferation and differentiation of organ-intrinsic precursor or stem cells) and angiogenic. The number of molecules known to mediate the paracrine action of MSCs increases every day; some of these, which may fit more than one category, are discussed in detail below and summarized in Table 1 and contextualized in Fig. 3. The ability to preferentially dock at sites of injured tissues, which may be influenced by several factors (reviewed in [45]), adds to the regenerative properties of MSCs as it increases the likelihood of systemically delivered cells finding the areas where their paracrine effects are most needed, and this is of particular interest for clinical applications.

3.1. Homing of cultured MSCs

MSCs are known to migrate or dock preferentially to injured sites when infused in animal models of injury (reviewed in [10]), and this property can be attributed to the expression of growth factor, chemokine and extracellular matrix receptors on the surface of MSCs. In an *in vitro* assay, murine MSCs migrated toward cells isolated from bleomycin-injured mouse lungs, but not toward healthy lung cells [46]. Chemotaxis assays show that cultured MSCs migrate toward different growth factors and chemokines in a dose-dependent fashion *in vitro*, and that chemokine-driven migration is stimulated by the pro-inflammatory cytokine tumor necrosis factor (TNF)-α [47], which indicates that chemoattraction can direct systemically infused MSCs to inflammatory sites. Other mechanisms involved in MSC homing or docking include attachment to endothelium. Endothelial cells become activated under injury conditions, and this state is characterized by the expression of surface molecules that allow docking of circulating cells. Two of these molecules, vascular cell adhesion molecule 1 (VCAM-1, aka CD106) and E-selectin (CD62E), are ligands for the MSC surface molecules integrin α4/β1 (CD49d/CD29) and CD44, respectively [48,49]. Osteopontin, whose expression is upregulated in osteocytes under hypoxic conditions [50], and hyaluronan, whose expression is increased in the kidney after experimentally induced acute renal failure [51], also can retain systemically infused MSCs via binding of CD44, which highlights the importance of this molecule for MSC homing and docking. The irony regarding CD49d and CD44 is that these two molecules are sensitive to the proteolytic action of trypsin [52], the enzyme most widely used to harvest MSCs from culture plates.

3.2. Main soluble factors secreted by MSCs

3.2.1. Anti-apoptosis

In a scenario where MSCs are administered with the aim of treating acute lesions, the first expected effect is the reduction of the extent of cell death, and this is observed in animal models of tissue injury and in co-culture experiments. Togel et al. reported that infused MSCs attach to the renal microvascular circulation and decrease apoptosis of adjacent cells in a model of acute kidney injury [53]. In order to elucidate the factors responsible for the observed renoprotective effect, these authors analyzed the MSC-conditioned medium and verified the presence of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1), factors that

Table 1
Trophic and immunomodulatory factors secreted by cultured MSCs.

Effect	Molecule	Reference(s)
Anti-apoptotic	VEGF	[53,56]
	HGF	[53,56]
	IGF-I	[53]
	Stanniocalcin-1	[55]
	TGF-β	[56]
	bFGF	[56]
	GM-CSF	[56]
Immunomodulatory	PGE-2	[59,63,65,66,68]
	TGF-β	[57,63]
	HGF	[57]
	mpCCL2	[67]
	IDO	[69]
	iNOS	[68]
	HLA-G5	[70–72]
	LIF	[73,74]
Anti-scarring	bFGF	[81]
	HGF	[81]
	Adrenomedullin (?)	[82]
Supportive	SCF	[83,84]
	LIF	[83,84]
	IL-6	[83,84]
	M-CSF	[83,84]
	SDF-1	[85,86]
	Angiopoietin-1	[86]
Angiogenic	bFGF	[89]
	VEGF	[89,90]
	PlGF	[89]
	MCP-1	[89,90]
	IL-6	[90]
	Extracellular matrix molecules	[91]
Chemoattractant	CCL2 (MCP-1)	Reviewed in [10]
	CCL3 (MIP-1α)	
	CCL4 (MIP-1β)	
	CCL5 (RANTES)	
	CCL7 (MCP-3)	
	CCL20 (MIP-3α)	
	CCL26 (eotaxin-3)	
	CX3CL1 (fractalkine)	
	CXCL5 (ENA-78)	
	CXCL11 (i-TAC)	
	CXCL1 (GROα)	
	CXCL2 (GROβ)	
	CXCL8 (IL-8)	
	CCL10 (IP-10)	
	CXCL12 (SDF-1)	

enhance endothelial cell growth and survival. Parekkadan et al. found the presence of these and other anti-apoptotic molecules in MSC-conditioned medium and, interestingly, showed that an MSC-containing bioreactor connected to the bloodstream of rats experimentally subjected to fulminant hepatic failure resulted in the survival of 71% of the animals in contrast to 14% survival in the control group [54]. MSCs reduce apoptosis of UV-irradiated fibroblasts and lung epithelial tumor cells cultured under low pH and hypoxia, and the up-regulation and secretion of stanniocalcin-1 has been found to be at least partially responsible for this anti-apoptotic effect [55]. Also, adipose tissue-derived MSCs have been shown to express HGF, VEGF, transforming growth factor beta (TGF-β), basic fibroblast growth factor (bFGF, aka FGF2) and granulocyte–macrophage colony-stimulating factor (GM-CSF), and the expression of these molecules was found to increase under hypoxic culture conditions; particularly, VEGF upregulation under hypoxia has been shown to be greater than that observed for other factors [56]. Hypoxia takes place in the first stages of tissue injury, and secretion of anti-apoptotic factors by MSCs at this stage minimizes the extent of cell death in the tissues surrounding the injured areas; accordingly, in the latter study, it was further demonstrated that cultured, adipose-derived MSCs reduce necrosis and improve perfusion when injected into mice experimentally subjected to hind limb ischemia [56]. We suggest that this anti-apoptotic activity could serve to limit the field of injury in *in vivo* circumstances.

3.2.2. Immunomodulation

The realization that cultured MSCs have immunomodulatory properties comes from experiments where they were shown to directly inhibit the proliferation of αβ T cells *in vitro* [57,58]. More recently, MSCs have been shown to suppress γδ T cells as well [59]. In addition, MSCs have been found to escape cytotoxic T cell-mediated lysis [60]. To date, MSCs are known to affect not only T cells, but also other cells of the immune system. MSCs can inhibit [61] or promote [62] B cell proliferation, suppress NK cell activation [63,64], and modulate the cytokine secretion profile of dendritic cells [65] and macrophages [66]. The ability to interact with dendritic cells and macrophages provides MSCs with extended indirect influence on the immune system. Whereas most of the molecular mechanisms that mediate the MSC suppressive effect on B cells remain unknown, a CCL2 variant resulting from modification of CCL2 by matrix metalloproteinases has been shown to suppress immunoglobulin production by plasma cells, and transplantation of syngeneic MSCs into hemophilic mice with pre-developed anti-human factor VIII (hFVIII) antibodies resulted in a robust decrease in hFVIII-specific IgG levels [67].

Secreted prostaglandin E2 (PGE-2) is a central mediator in many of the effects of MSCs on immune cells, as inhibitors of the synthesis of this molecule diminish MSC-driven anti-proliferative effects on T [59,65,68] and NK cells [63], and PGE-2 is involved in the modulation of the secretory profile of dendritic cells [65] and macrophages [66]. Neutralizing antibodies to TGF-β1 have been reported to impair the immunomodulatory effects of MSCs on T [57] and NK cells [63]. Likewise, neutralizing antibodies to HGF have revealed that this molecule also mediates MSC anti-proliferative effects on T cells [57]. Degradation of tryptophan as a consequence of expression of indoleamine 2,3-dioxygenase (IDO) by MSCs in co-cultures also has been shown to halt T cell proliferation [69], and tryptophan catabolites such as L-kynurenine and picolinic acid have been previously shown to inhibit activation of CD4+ and CD8+ T cells and, to a lesser extent, of NK cells independent of the presence of tryptophan [70].

Sato et al. focused on the expression of inducible nitric oxide synthase (iNOS) by murine MSCs and found that inhibition of this enzyme abolishes MSC anti-proliferative effect on T cells, but failed to obtain the same result with neutralizing antibodies to TGF-β or IDO [68]. It is important to emphasize that this seeming discrepancy between some of the results obtained by Sato et al. and the others shown above may reflect species-specific differences, as all other studies mentioned earlier in this section used human cells. Other molecules that mediate immunomodulatory effects of MSCs include interleukin (IL)-10, human leukocyte antigen G (HLA-G) [70–72] and leukemia inhibitory factor (LIF) [73], the latter playing an important role not only in the suppression of T cell proliferation, but also in the generation and maintenance of regulatory T cells [74].

The consequences of the mechanisms involved in the immunomodulatory effects of cultured MSCs discussed herein can be observed *in vivo*, although in most cases defining the molecules responsible for the observed immunomodulation is hindered by the complexity of the interactions between MSCs and endogenous cells, which may activate the host's intrinsic mechanisms. Le Blanc et al. have shown that MSC infusions are effective in treating cases of severe, steroid-refractory Graft versus Host Disease (GvHD) in humans [75]. In rats, MSC-conditioned medium was found to reduce the migration of adoptive leukocytes to the liver after

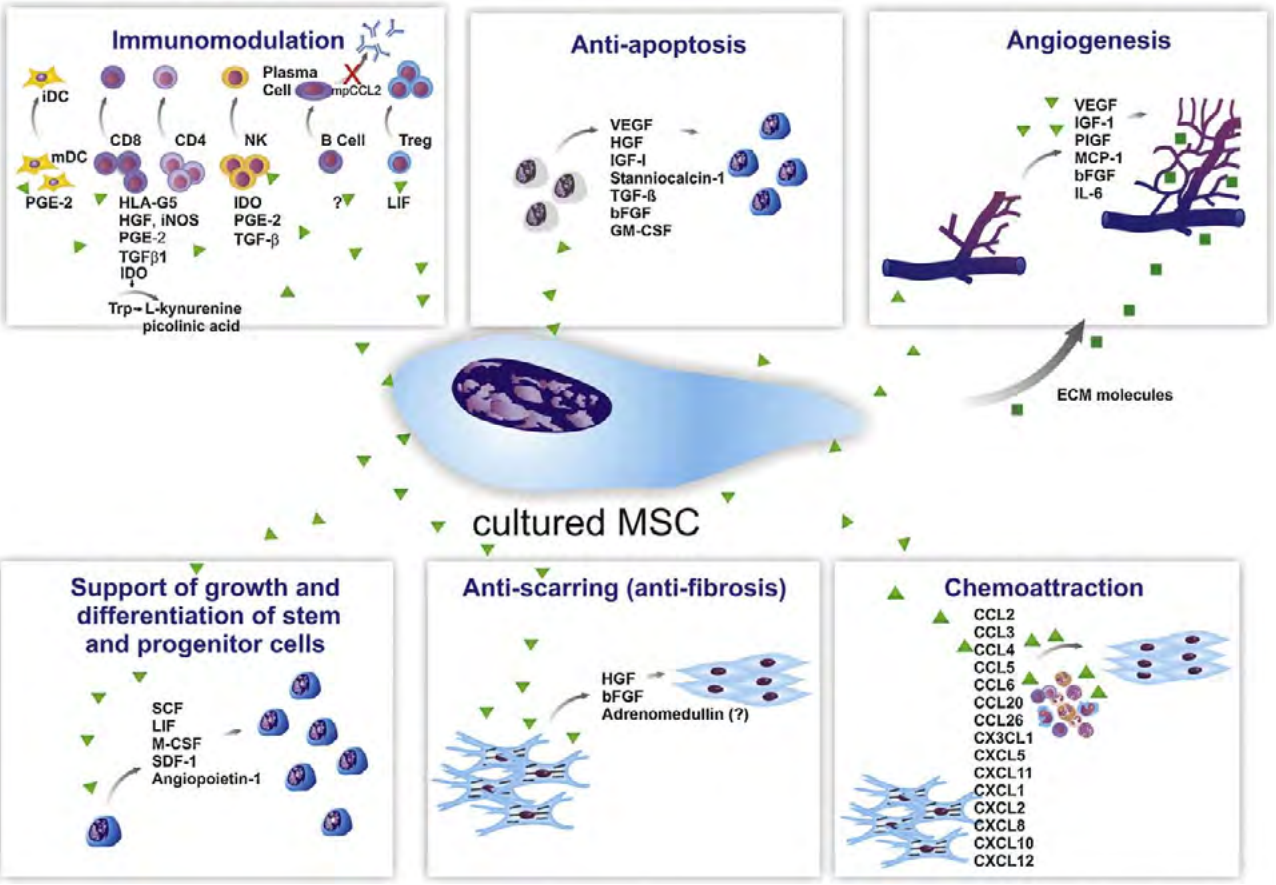

Fig. 3. Paracrine effects of cultured MSCs. The secretion of a broad range of bioactive molecules is now believed to be the main mechanism by which MSCs achieve their therapeutic effect and it can be divided into six main categories: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring and chemoattraction. Although the number of molecules known to mediate the paracrine action of MSCs increases every day, several factors that have been shown to be secreted by cultured MSC are depicted here for illustrative purposes. The immunomodulatory effects of MSCs consist of inhibition of the proliferation of CD8+ and CD4+ T lymphocytes and natural killer (NK) cells, suppression of immunoglobulin production by plasma cells, inhibition of maturation of dendritic cells (DCs) and stimulation of the proliferation of regulatory T cells. The secretion of PGE-2, HLA-G5, HGF, iNOS, IDO, TGF-β, LIF and IL-10 contributes to this effect. MSCs also limit apoptosis, and the principal bioactive molecules responsible for this are VEGF, HGF, IGF-I, stanniocalcin-1, TGF-β and GM-CSF. In addition, MSCs stimulate local angiogenesis by secretion of extracellular matrix molecules, VEGF, IGF-1, PIGF, MCP-1, bFGF and IL-6, and also stimulate mitosis of tissue-intrinsic progenitor or stem cells by secretion of SCF, LIF, M-CSF, SDF-1 and angiopoietin-1. Moreover, HGF and bFGF (and, possibly, adrenomedullin) produced by MSCs contribute to inhibition of scarring caused by ischemia. Finally, a group of at least 15 chemokines produced by MSCs can elicit leukocyte migration to the injured area, which is important in normal tissue maintenance.

experimentally induced fulminant hepatic failure [54]. In mice subjected to bleomycin-induced lung injury, levels of mRNA for the pro-inflammatory cytokines IL-1β, IL-2 and interferon-γ (IFN-γ) are reduced after systemic MSC administration [46]. In a murine model of lipopolysaccharide-induced acute lung injury, the expression of genes coding for the pro-inflammatory cytokines TNF-α, IL-1α and IL-1β decreased 8.6-, 3.6- and 3.2-fold, respectively, after systemic MSC infusion; conversely, IL-1 receptor antagonist expression showed a 2.2-fold increase [76].

Conversely, the MSC effects on the immune system may not be strictly suppressive. For example, low numbers of MSCs can render dendritic cells prone to promoting T cell activation whereas high numbers of MSCs are required to cause the opposite effect [77]. Low doses of IFN-γ allow MSCs to express class II major histocompatibility complex (MHC) molecules and behave as antigen-presenting cells [78]; on the other hand, higher doses of IFN-γ lead to a decrease in the surface levels of class II MHC molecules [78] and secretion of anti-inflammatory factors [79]. Furthermore, MSCs support B cell expansion and differentiation [62]. These findings indicate that the behavior of MSCs toward the immune system is context-sensitive.

We have recently proposed a model in which pericytes play an active role during tissue repair [10]. According to our model, focal injury leads to local cell death; inflammatory cells migrate into the damaged area; pericytes become activated (i.e., undergo a change in gene expression and become proliferative) and secrete different bioactive molecules that act in concert to resolve the lesion. It is likely that, at the early steps of this process, these pericyte-derived cells provide a supportive effect on immune cells. Consistent with this view, cultured retinal pericytes have been shown to express pro-inflammatory molecules that are chemoattractant to inflammatory cells, namely eotaxin (aka CCL11), granulocyte colony-stimulating factor (G-CSF), IL-8 and regulated upon activation, normal T cell expressed and secreted (RANTES, aka CCL5), when insulted by exposure to a high glucose concentration [80]. In the same study, exposure of pericytes to the pro-inflammatory molecules TNF-α or IL-1β for 24 h resulted in an increase in the expression of many molecules known to be chemoattractive and stimulatory for different immune cells, including IL-1β, IL-6, IL-7, IL-12, IL-16, IL-1 receptor antagonist (IL-1ra), TNF-α, TNF-β, epithelial neutrophil-activating protein 78 (ENA-78, aka CXCL5), eotaxin, IL-8 (aka CXCL8), monocyte chemoattractant protein 1 (MCP-1, aka CCL2), macrophage inflammatory protein (MIP)-1α (aka CCL3), MIP-1β (aka CCL4), RANTES, intercellular adhesion molecule-1 (ICAM-1), VCAM-1, G-CSF, GM-CSF, growth hormone, stem cell factor (SCF), $VEGF_{165}$, bFGF, thyroid-stimulating hormone (TSH), CD40 and CD40 ligand. This suggests that pericytes respond to inflammatory cells at the early stages of wound healing and

provide physiological support for the subsequent steps of the immune response. However, as the local environment undergoes changes during the healing process, it is likely that the expression profile of pericytes changes with time. For example, perivascular cells have been shown to proliferate and secrete HGF only 3 days after induction of an ischemic lesion in murine adipose tissue ([81]; see below). As the pericyte changes, it becomes an MSC-like cell that inhibits the immunosurveillance of the injury site and prevents the initiation of autoimmune events.

3.2.3. Anti-scarring

Although the anti-fibrotic effects of cultured MSCs have been demonstrated in different animal models, the molecular mechanisms behind this effect are not yet fully understood. Based on studies done to date, in most cases MSC administration is effective only if it takes place before the establishment of massive fibrosis (reviewed in [8]). Recently, bFGF and HGF have been shown to be involved in the prevention of fibrosis in a murine model of ischemia-reperfusion of adipose tissue [81]. In that study, human and murine BM-derived MSCs, human adipose-derived stem cells and dermal fibroblasts were found to express HGF in response to stimulation with bFGF, an effect that could be blocked by a c-Jun N-terminal kinase (JNK) inhibitor. Administration of either a neutralizing antibody against bFGF or a JNK inhibitor to the injured adipose tissue resulted in impaired proliferation of CD34+CD31-stromal/perivascular cells (viewed here as MSCs) and a reduction of severe post-lesion fibrogenesis; on the other hand, a neutralizing antibody against HGF did not stop proliferation of CD34+CD31-stromal/perivascular cells, and this resulted in marked fibrogenesis. These results reveal that, in a situation of tissue injury, perivascular cells become proliferative and secrete HGF, which in turn mediates anti-fibrotic (and immunomodulatory) effects. Administration of culture-expanded MSCs to prevent fibrosis can, thus, be viewed as a way to augment local production of HGF (and probably other anti-scarring factors) in cases where fibrosis is to be avoided. Lastly, in a rat model of global heart failure, it was shown that transplanted MSCs decreased cardiac fibrosis; the secretion of adrenomedullin appears to be one of the factors which mediate this anti-fibrotic effect [82].

3.2.4. Support to the growth and differentiation of local stem and progenitor cells

Cultured MSCs support hematopoiesis *in vitro*, and this ability involves the constitutive secretion of soluble factors such as SCF, LIF, IL-6, and macrophage colony-stimulating factor (M-CSF); in addition, hematopoietic support can be further augmented by IL-1α-induced secretion of G-CSF and GM-CSF [83,84]. *In vivo*, adventitial reticular cells, which are putative MSCs *in vivo* [42], sustain the hematopoietic stem cell pool through the secretion of stromal-derived factor 1 (SDF-1, aka CXCL12) [85], which suggests that the hematopoietic support observed *in vitro* mimics some aspects of that observed under physiological conditions *in vivo*. In a situation of ischemic injury in murine brain, cells in blood vessels were shown to express SDF-1 and angiopoietin-1, which recruited and supported neural precursors [86]. Expression of SDF-1 and angiopoietin-1 is characteristic of pericytes [87,88], which indicates that these cells were responsible for the observed recruitment and support of neural progenitors.

3.2.5. Angiogenesis

Angiogenic support provided by MSCs can be considered one more supportive effect as discussed above, and it is treated separately here because re-establishment of blood supply is fundamental for recovery of damaged tissues. The pro-angiogenic effect of MSCs has been demonstrated in a murine model of hind limb ischemia [89]. In that study, the authors detected bFGF, VEGF, placental growth factor (PlGF), and MCP-1 in MSC-conditioned medium, and also verified the presence of bFGF and VEGF around the infused cells *in situ* after local administration. Hung et al. also demonstrated that MSC-conditioned medium contains high amounts of angiogenic and anti-apoptotic factors such as IL-6, VEGF and MCP-1, which inhibit the death of endothelial cells cultured under hypoxic conditions and promote the formation of capillary-like structures in an *in vitro* assay [90]. Recently, some populations of BM-derived MSCs have been shown to support the formation of vessel-like structures by endothelial cells *in vitro* in a medium devoid of the angiogenic factors VEGF-A, bFGF and IGF-1; in this system, MSCs provide, in addition to soluble angiogenic factors, extracellular matrix components that serve as a substrate for endothelial cells [91]. Lastly, the transition of MSCs back to pericytes situated on newly formed vessels serves to stabilize the forming vasculature both *in vitro* [91] and *in vivo* [92].

3.2.6. Chemoattraction

Cultured MSCs secrete a variety of chemoattractant molecules, which include CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL20 (MIP-3α), CCL26 (eotaxin-3), CX3CL1 (fractalkine), CXCL5 (ENA-78), CXCL11 (i-TAC), CXCL1 (GROα), CXCL12 (SDF-1), CXCL8 (IL-8), CXCL2 (GROβ) and CXCL10 (IP-10) (reviewed in [10]). Target cells for these include monocytes, eosinophils, neutrophils, basophils, memory and naïve T cells, B cells, NK cells, dendritic cells and hematopoietic and endothelial progenitors [93]. Although these molecules are constitutively expressed by cultured MSCs, with some discrepancies between studies most probably reflecting different culture conditions, it is likely that the pattern of chemokine expression by MSCs is modified by exposure to other cell types, particularly immune cells.

4. Consequences for the clinic

The overview discussed herein indicates that MSCs are promising tools for the treatment of different types of conditions, because they secrete a multitude of bioactive molecules that ultimately lead to reformation of tissues at sites of injury. In many instances, the time required to isolate and culture-expand MSCs to increase cell numbers precludes the immediate application of autologous MSCs to cases of acute injury, e.g., ischemic lesions. Given that long-term engraftment is not necessarily required for MSCs to exert most of their therapeutic effects, this hurdle could be overcome by banking third-party MSCs so that they are readily available when necessary. Since MSCs are present in different tissues due to their association with blood vessels, every piece of vascularized tissue could be used as a source of MSCs. Considering the data showing that MSCs from different tissues are not identical, further studies comparing MSCs obtained from different tissue sources are necessary to determine if MSCs could be more effective for specific applications as a function of their site of origin.

The alternative to the use of cultured MSCs is the isolation of fresh MSCs, which in our view are pericytes; however, there are no data available to determine if pericytes and cultured MSCs share the same properties. Also, it is possible that the frequency of bona fide MSCs *in vivo* is low, which again points to *ex vivo* expansion. The Cell Therapy Laboratory at National Institute of Science and Technology for Stem Cells and Cell Therapy (Ribeirão Preto, Brazil) currently produces good manufacturing practice (GMP)-grade MSCs to experimentally treat GvHD patients. One of the problems with the culture technology currently used (serial passaging in plastic flasks) is that it is inefficient albeit effective. To minimize this difficulty, the scale-up of MSC production in bioreactors is under development in this Institute and elsewhere.

In spite of the fact that some of the mechanisms involved in the therapeutic effects of MSCs are known today, further research

focusing on this topic is required to devise strategies aimed at increasing the efficiency of therapies not only for GvHD but also for other conditions. Pre-treatment of cultured MSCs with soluble factors such as IFN-γ, for example, could enhance the treatment of certain immunological diseases. The efficiency of MSC-based therapies could also be increased by subjecting cultured MSCs to hypoxic conditions before administration to patients. Screening for enzymes that can efficiently harvest cultured MSCs while preserving molecules responsible for docking systemically infused cells at injured sites may also prove valuable for therapeutic purposes. Lastly, the targeting of MSCs to precise locations of injury to deliver optimized doses of cells is the next technological frontier to be overcome.

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08

ARTICLE

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

Pietro Gentile

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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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Abstract: 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. 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. 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 hair follicles.

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, 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

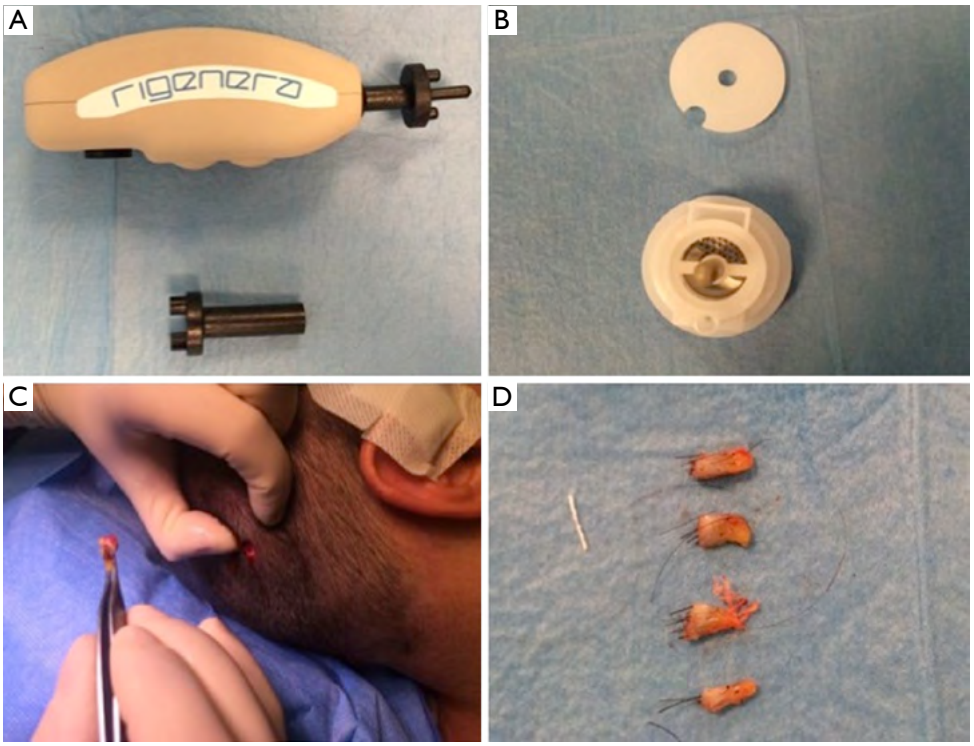

Figure 1 Rigenera procedure phase 1 (punch biopsy and cutting of scal tissues). (A) Rigenera Securdrill Device; (B) Rigeneracons kit; (C) the extraction of the scalp tissues during punch biopsy; (D) the authors cut the scalp tissues into the strips (2 mm × 2 mm).

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 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

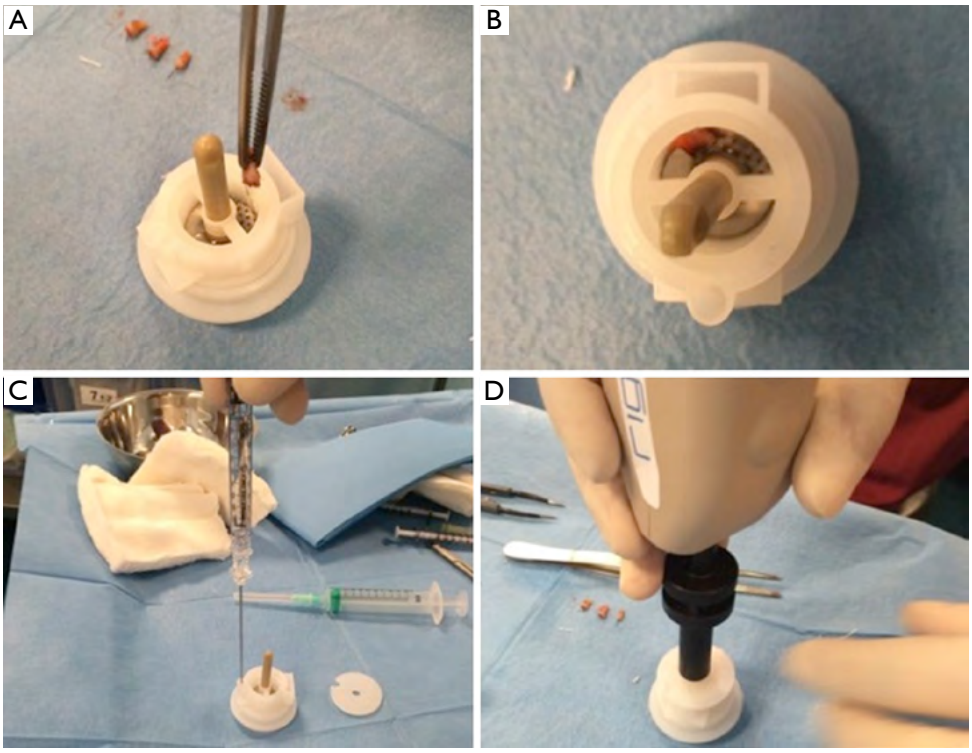

Figure 2 Rigenera procedure phase 2 (positioning of scalp tissue in Rigeneracons and centrifugation). (A) The strips collected into Rigeneracons; (B) deatil of Rigeneracons containing one strip; (C) the addition of 1.2 mL of physiologic solution; (D) centrifugation at 80 RPM with Rigenera Securdrill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

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).

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).

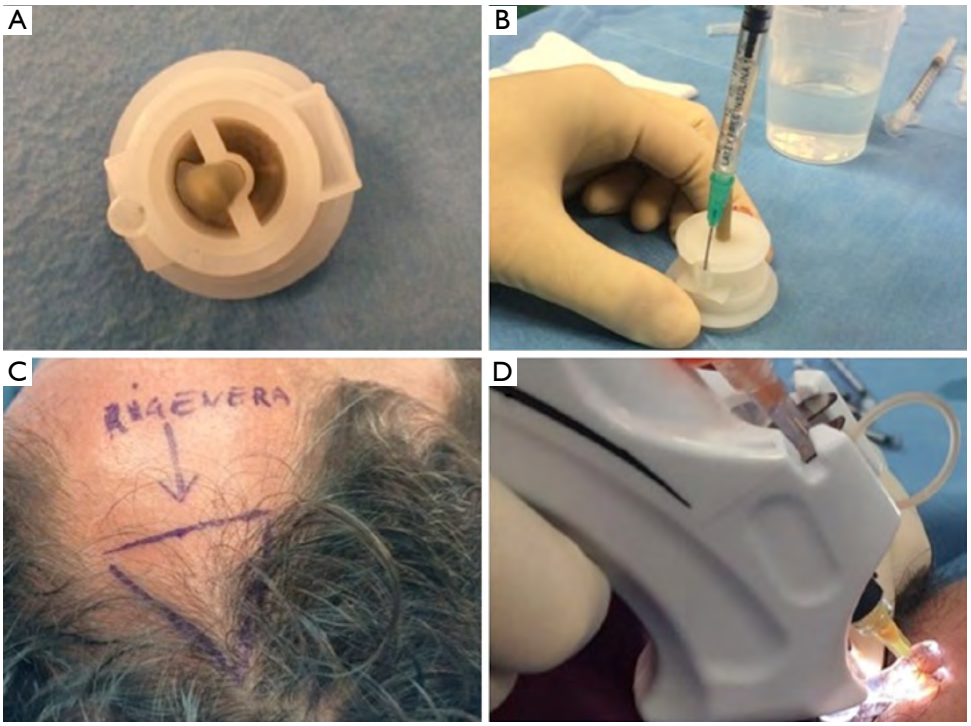

Figure 3 Rigenera procedure phase 3 (cell suspension contained in Rigeneracons and infiltration). (A) The cell suspension obtained by the system contained in Rigeneracons; (B) harvesting of cell suspension; (C) the selected area of the scalp treated; (D) mechanical and controlled infiltration performed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

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 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,

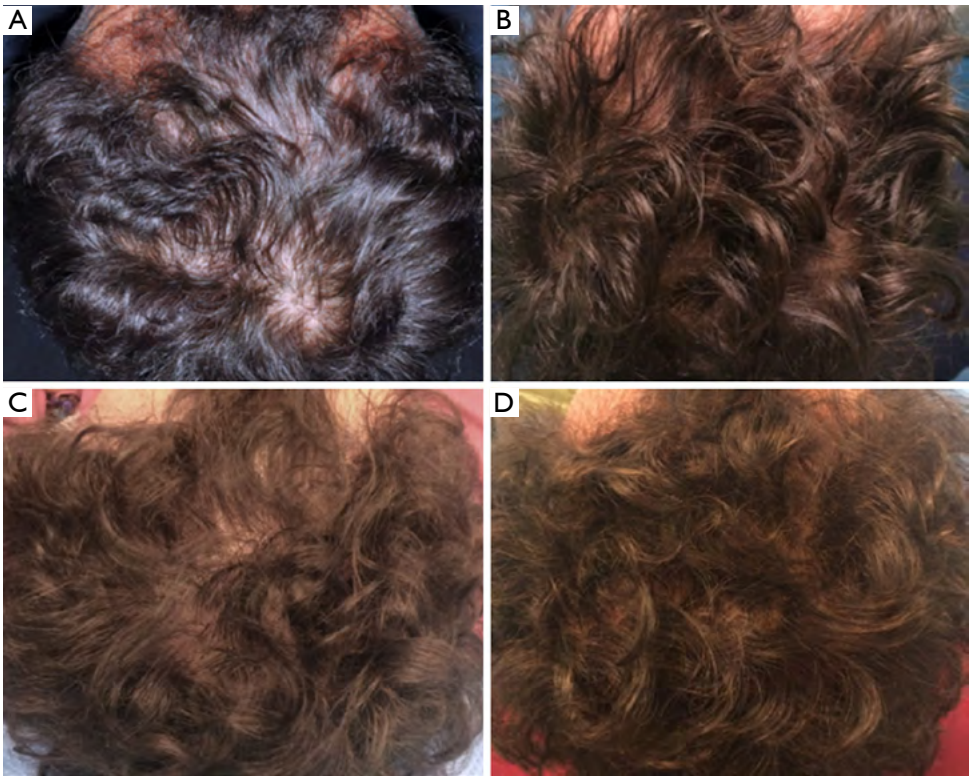

Figure 4 A smoker 45-year-old male patient affected by androgenetic alopecia classified AGA 3 according to Nordwood-Hamilton scale. (A) Preoperative situation at T0 of the scalp with hair loss localized to the parietal, temporal and frontal areas; (B) postoperative situation of the scalp at T1 after 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.

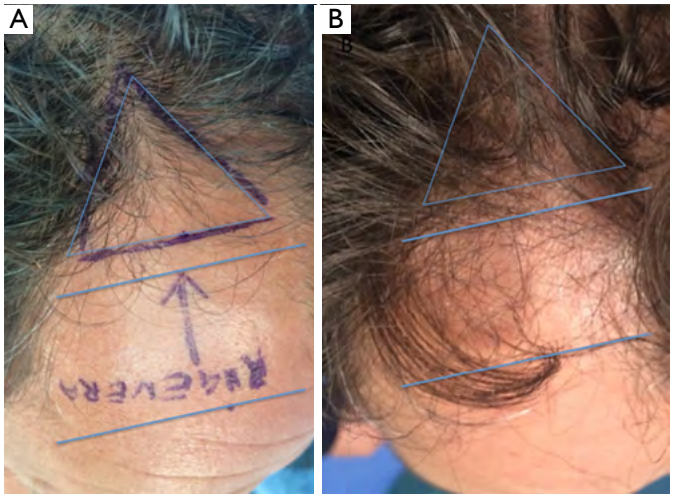

Figure 5 Detail of temporal right area of male patient affected by androgenetic alopecia classified AGA 3 according to Nordwood-Hamilton scale. (A) Preoperative situation at T0 of the scalp with hair loss localized to the right temporal and frontal area identified by blue lines; (B) postoperative situation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

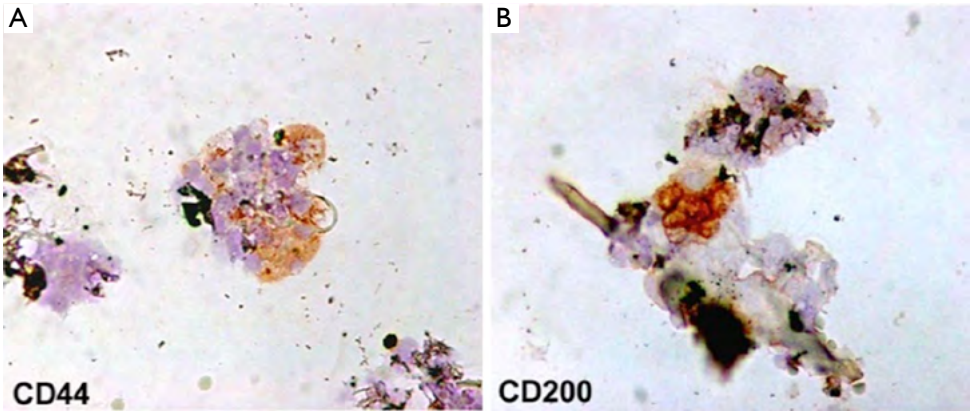

Figure 6 Immunophenotypic characterization of hair follicle stem cells in human scalp tissue suspension. Immmunocytochemistry for CD44 and CD200 stem cell markers. (A) hair follicle-derived mesenchymal stem cells; (B) hair follicle epithelial stem cells. Original magnification 400×.

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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09

ARTICLE

Dermoscopy is the Crucial Step for Proper Outcome Prospection when Treating Androgenetic Alopecia with the Regenera® Protocol: a Score Proposal.

Pinto H, Gálvez R, Casanova J.

Dermoscopy is the Crucial Step for Proper Outcome Prospection when Treating Androgenetic Alopecia with the Regenera® Protocol: a Score Proposal.

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Abstract

There is no actual treatment capable of creating hair follicles *de novo,* since a hair follicle is not a single tissue but a tiny, full anatomical structure. The Regenera® method is based on the regeneration and recovery of severely non-functional but still-alive hair follicles. For 1.5 years, clinical assessment has been the sole pillar of any outcome prospection issued. Wrong prospections could be affecting about 10% of patients expecting good results. Scalp dermoscopy was then included in routine diagnosis tests of every patient willing to be treated with the Regenera® protocol. When analyzing the data of the dermoscopy of patients with a poor prospection, it was clear that the expectancies related to the treatment´s results were overestimated.

MeSH words. Regenera, Androgenetic Alopecia, Score, Hair loss.

Introduction

Hair restoration treatments claim to increase the number of follicles and to be able to improve hair general conditions, its health or appearance. Hair transplant aside, the truth is there is no actual treatment capable of creating hair follicles *de novo,*since a hair follicle is not a single tissue but a tiny, full anatomical structure. The Regenera® method is no exception to this rule, and its action is based on the regeneration and recovery of severely non-functional but still-alive hair follicles. These are called "miniaturized" follicles and, though their presence is not pathognomonic of Androgenetic Alopecia (AA), these hypotrophic and hypofunctioning follicles are naturally found in the course of its evolution. The amount of miniaturized hair follicles is in direct relationship with the severity of AA, which is perfectly characterized by the Hamilton-Norwood and the Ludwig scales. During the initial AA stages, a fair amount of miniaturized hair follicles and units that share miniaturized and non-miniaturized hairs can be expected. The advance of AA involves a shift from miniaturized and non-miniaturized multiple hair units to single hair units. An increasing but variable amount of miniaturized hair follicles is expected, but cannot be analyzed seriously without proper evaluation of the patient as a whole. The more miniaturized hair follicles a patient has at the beginning of the treatment, the better the expected results.

The Regenera® protocol

The Regenera® protocol uses a special microdermatome (Regeneracons®) that breaks up the structure of skin by means of 2.5-mm biopsies and filters elements smaller than 50 micras,[4] such as cells. The processed material is reinjected into the patient's skin at the scalp. Fiber proteins and the stratum corneum are naturally excluded, since the cell fraction obtained is the biological product desired with therapeutic action. No added chemicals or any other physical processing medium is used. The Regenera® protocol involves the following sequential main steps: i) Local anesthetic—Lidocaine 2% without adrenaline—, ii) Skin micrograft from the napearea—2.5 mm punch x 3—, iii) Micrograft placed in the Regeneracons® and in the machine —mechanical deaggregation at 80 rpm for 1 minute—, v) Recovery of the filtered cells, and vi) Deep intradermal injection.

Tackling the problem

For 1.5 years, clinical assessment has been the sole pillar of any outcome prospection issued. Thus, certain clinical conditions that used to be thought as" non-favorable" slowly got established as actual contraindications or exclusion criteria for the Regenera® method: systemic pathologies, local scalp pathologies, mid or severe Androgenetic Alopecia (Hamilton Score >3, Ludwig Score >1), psychological stress, and unrealistic expectancies. In most patients, the issued prospection was accurate or even extremely accurate when it came to foresee a poor outcome. Still, and on the other hand, we had some cases that predicted excellent results from a clinical point of view, but fail to achieve them on treatment delivery.

To overcome this obstacle, one single fact had to be acknowledged: there was a very small but constantly growing empiricism of wrong prospections that could be affecting about 10% of patients expecting good results (based on the clinical assessment). At that point, it seemed obvious to us that there was something that we were missing.

Scalp dermoscopy was immediately included in routine diagnosis tests of every patient willing to be treated with the Regenera® protocol and, soon enough, we found the answer to our problem. When analyzing the data of the dermoscopy of the patients with a poor prospection, it was clear that the expectancies related to the treatment´s results were overestimated. We were considering only two different scenarios when in fact, we were facing three (Table 1): inappropriate clinical characteristics (contraindication), clinical-dermoscopic consistency (majority of cases, accurate

prospection) and clinical-dermoscopic inconsistency (small amount of cases, not accurate prospection).

	Good Expectancy Dermoscopy	**Bad Expectancy Dermoscopy**
Good Expectancy Clinical Assessment	Accurate prospection	Not accurate prospection
Bad Expectancy Clinical Assessment	Contraindication	Contraindication

Table 1. The consistency between dermoscopy and clinical assessment expectancies affects therapeutic behavior, treatment results and prospection.

Since a Bad Expectancy Clinical Assessment would automatically imply the contraindication of the patient, the odds of mis-prospection were naturally reduced to Good Expectancy Clinical Assessment scenarios. The key to secure the prospection was to establish easy-to-follow criteria that would clearly define good and bad expectancies in terms of the dermoscopic analysis (allowing physicians to distinguish them).

Building the score

One hundred dermoscopies were evaluated. From the large amount of dermoscopic parameters assessed, eight signs stood out as very important (Table 2) and were finally included in the score (Table 3).A Firefly DE300 dermatoscope, Firefly Global®, Belmont, USA, was used as per technical specifications. Pictures were taken with a 40X zoom.

Sign		**Comment**
#1	Number of hairs	Generally (with a 40X zoom), we see a lot of follicles in any patient. The number of follicles diminishes according to the advance of AA.
#2	Distance between follicles	It is related to #1, reinforces the concept of an incipient condition and gives a sense of the amount of follicles that might benefit from the procedure. The distance between follicles grows with the advance of AA.

#3	Miniaturized follicles	They are the target of the Regenera® protocol. Generally, there are a lot of miniaturized follicles on the edges of the areas where AA is advancing. Still, careful evaluation is recommended, since this is a very important but tricky parameter.
#4	Hair diameter	The presence of different hair diameters is indicative of AA advance and of hair follicle involution.
#5	Miniaturized and non-miniaturized hairs in the same follicular unit	This is a typical sign that can be seen with AA's standard evolution. Miniaturization process takes place at different moments in every follicle, even within the same follicular unit.
#6	Yellowish pigment	Follicular openings with keratotic material and/or sebum.
#7	Inflammation	Inflammatory infiltrate can be seen as a ring around the shaft, at the point where the hair merges. It is 100% not specific, but it helps to discard local damage, infectious or inflammatory conditions.
#8	Scalp general condition	A bright, white pearl-like color and absence of desquamation are good signs.

Table 2. Dermoscopy findings included in the score.

Patients were classified into four groups based on the dermoscopy findings: Ideal outcome (A), Good outcome(B), Bad outcome(C), and Extremely Bad outcome(D). Standard dermoscopic images expected for these 4 groups are shown in Figure 1.

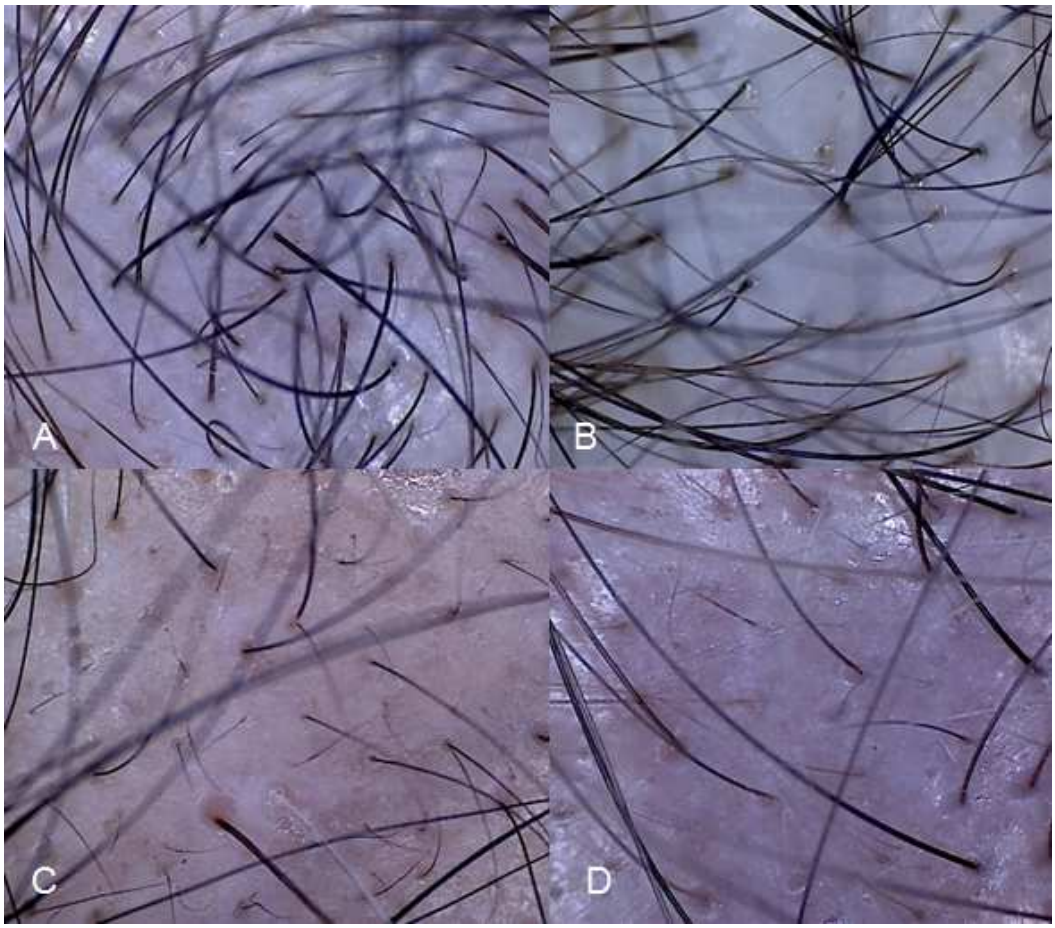

Figure 1. Patient classification based on the dermoscopy image. A: Ideal outcome, B: Good outcome, C: Bad outcome, and D: Very Bad outcome.

The evolution of the 8 dermoscopy parameters for the AA pathogeny represents AA's slow progress from Figure 2A to Figure 2D. Most of the time, dermoscopy can be correlated with the patient's physical appearance (represented by the Hamilton-Norton and Ludwig scales), and it is very tight, except for those cases that actually justify the fruition of a dermoscopy and the ultimate implementation of this study. The standard physical-appearance pictures expected for the patients of these 4 groups are shown in Figure 2. The basic idea underlying these rough, simplified and arbitrary classifications is that any patient showing an A-B pattern of physical appearance will have a very good result prospection whenever he or she has an A-B dermoscopy pattern.

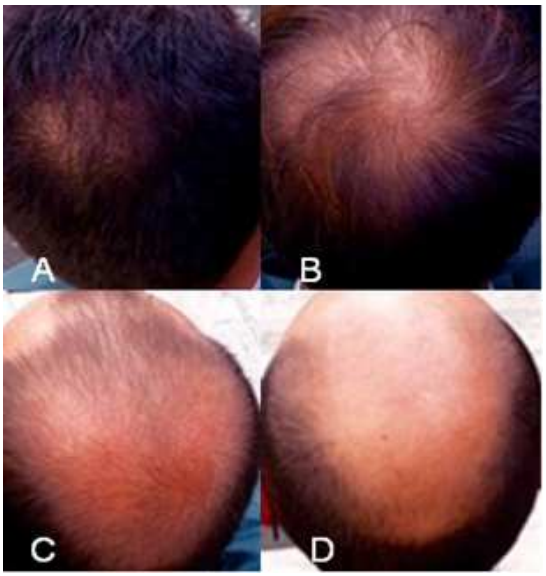

Figure 2. Patient classification based on appearance. A: Ideal outcome, B: Good outcome, C: Bad outcome, and D: Very Bad outcome.

Issuing a recommendation

After the dermoscopic analysis was performed, the score (Table 3) was run.

Sign		1 point	0 point
#1	Number of hairs	More than a few	A few
#2	Distance between follicles	Short	Mid to long
#3	Miniaturized follicles	A lot	A few
#4	Hair diameter	Diverse	All the same
#5	Miniaturized and non-miniaturized hairs in the same follicular unit	Yes	No
#6	Yellowish pigment	No	Yes
#7	Inflammation	No	Yes
#8	Scalp general condition	Good	Not good

Table 3. Proposed score.

Under a Good Expectancy Clinical Assessment (no contraindication scenarios), whenever the patient scored 6, 7 or 8 points, a good result prospection applied, and

when a 5 or less point score was achieved, a good result prospection did not apply. All these data together shaped the final indication and prospection algorithm (Figure 3) used today for the Regenera® method.

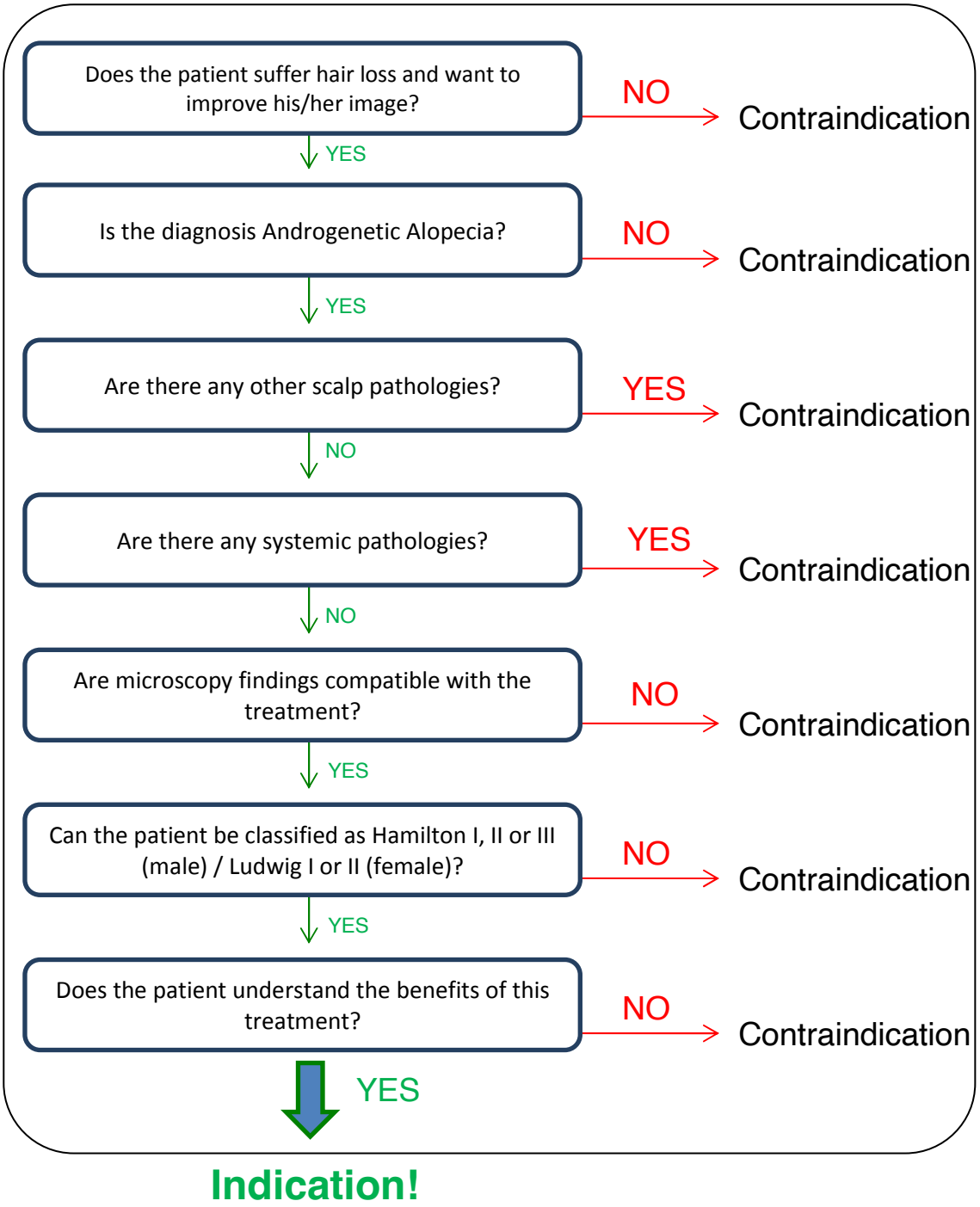

Figure 3. Patient indication and prospection algorithm.

10

ARTICLE

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

Valeria Purpura, Elena Bondioli, Antonio Graziano, Letizia Trovato, Davide Melandri, Martina Ghetti, Andrea Marchesini, Maria Gabriella Cusella De Angelis, Laura Benedetti, Gabriele Ceccarelli, Michele Riccio

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Video Article

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

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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**).

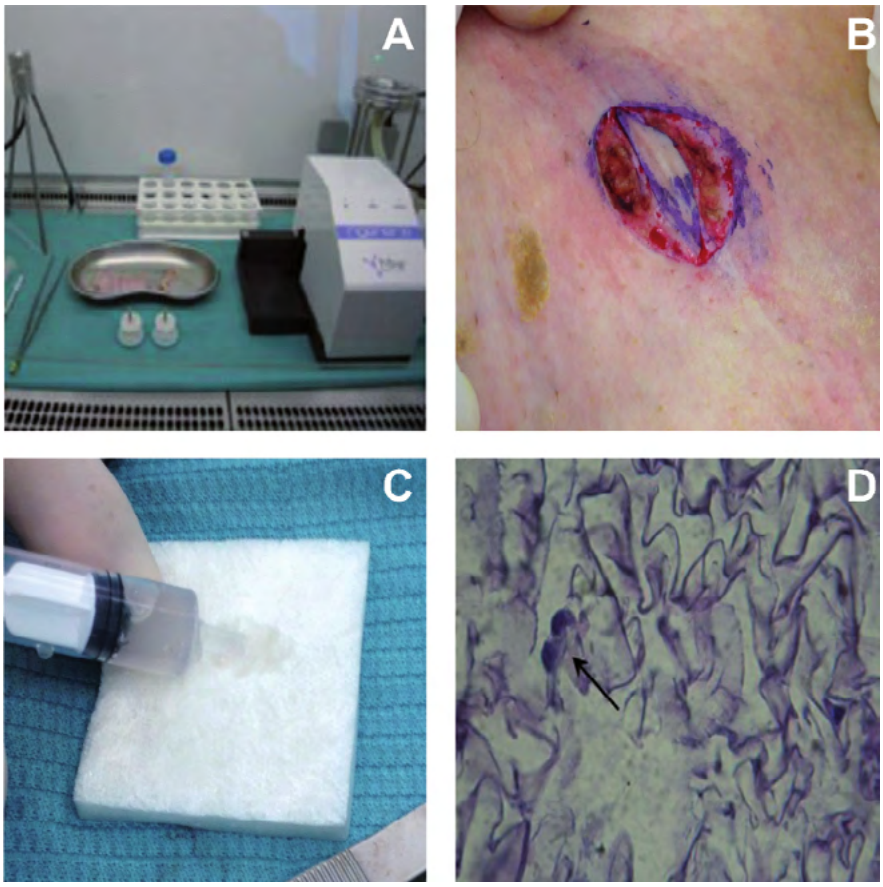


Figure 1. Bio-complexes Building Tissue Disrupting System (A) and Collection of a Small Piece of Derma from the Lesion Area of the Patient that was Subsequently Disaggregated with Tissue Disruptors. The bio-complexes were obtained combining the micro-grafts on collagen sponges to obtain human patches 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.

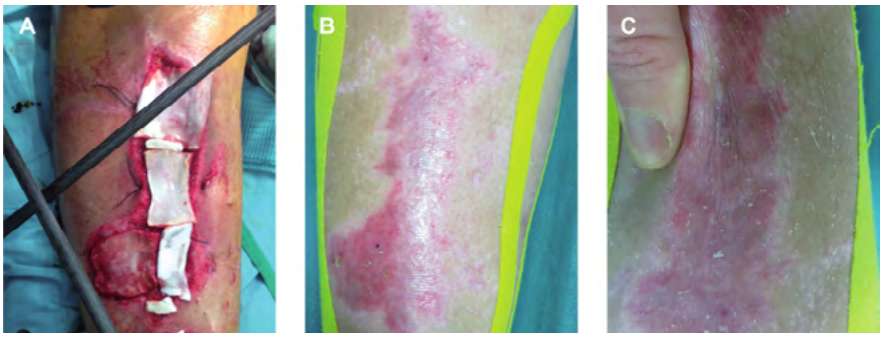


Figure 2. Bio-complexes Application on Leg Lesion The bio-complexes composed by collagen and micro-grafts obtained from the patient were applied on the leg lesion **(A)** and the wound was evaluated after 30 days **(B)** and 5 months **(C)**. Please click here to view a larger version of this figure.

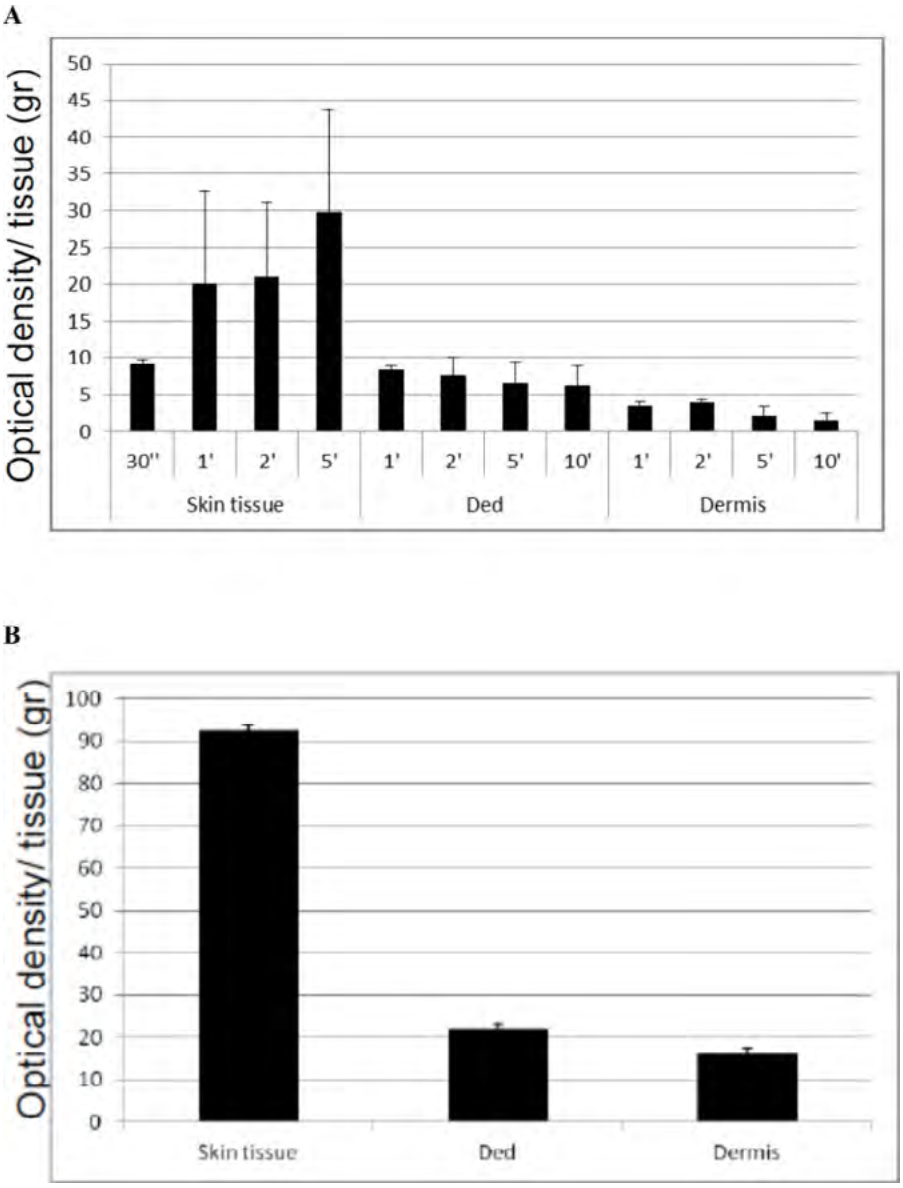


Figure 3. Cell Viability Immediately after Disaggregation at Different Times of Three Type of Cutaneous Tissues (A) with respect to Intact Cutaneous Tissues (B). The Skin, Ded and Dermis tissues derived from four different donors were disaggregated with tissue disruptors as indicate in the appropriate 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.

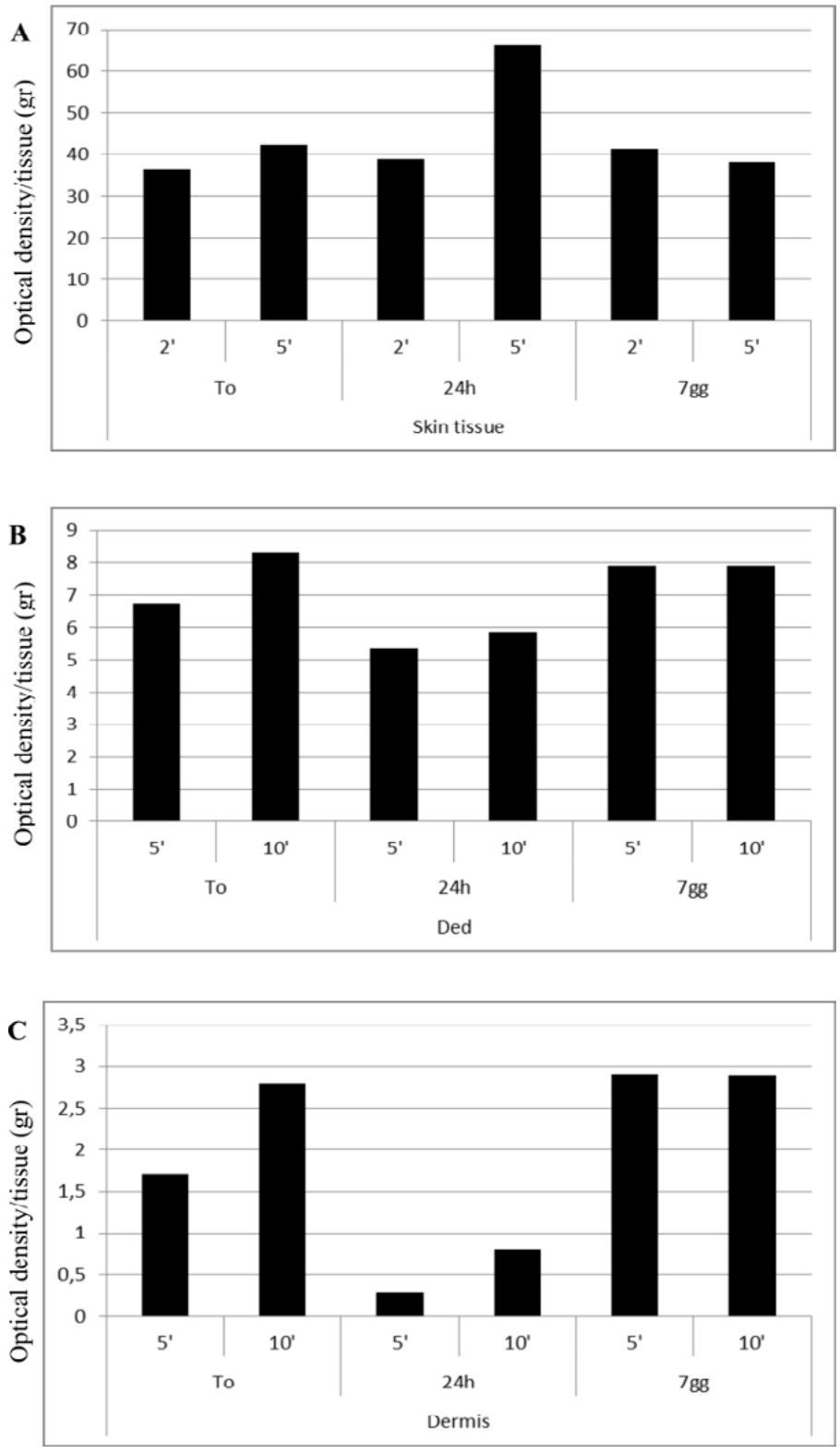


Figure 4. Cell Viability Levels of a Disaggregated Sample of Skin Tissue (A), Ded (B) and Dermis (C) to Starting Time (T_0) and after Subculture for 24 hr and 7 Days. The tissues were homogenized with tissue disruptors as indicate in the appropriate section in the text. The disaggregated samples were 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.

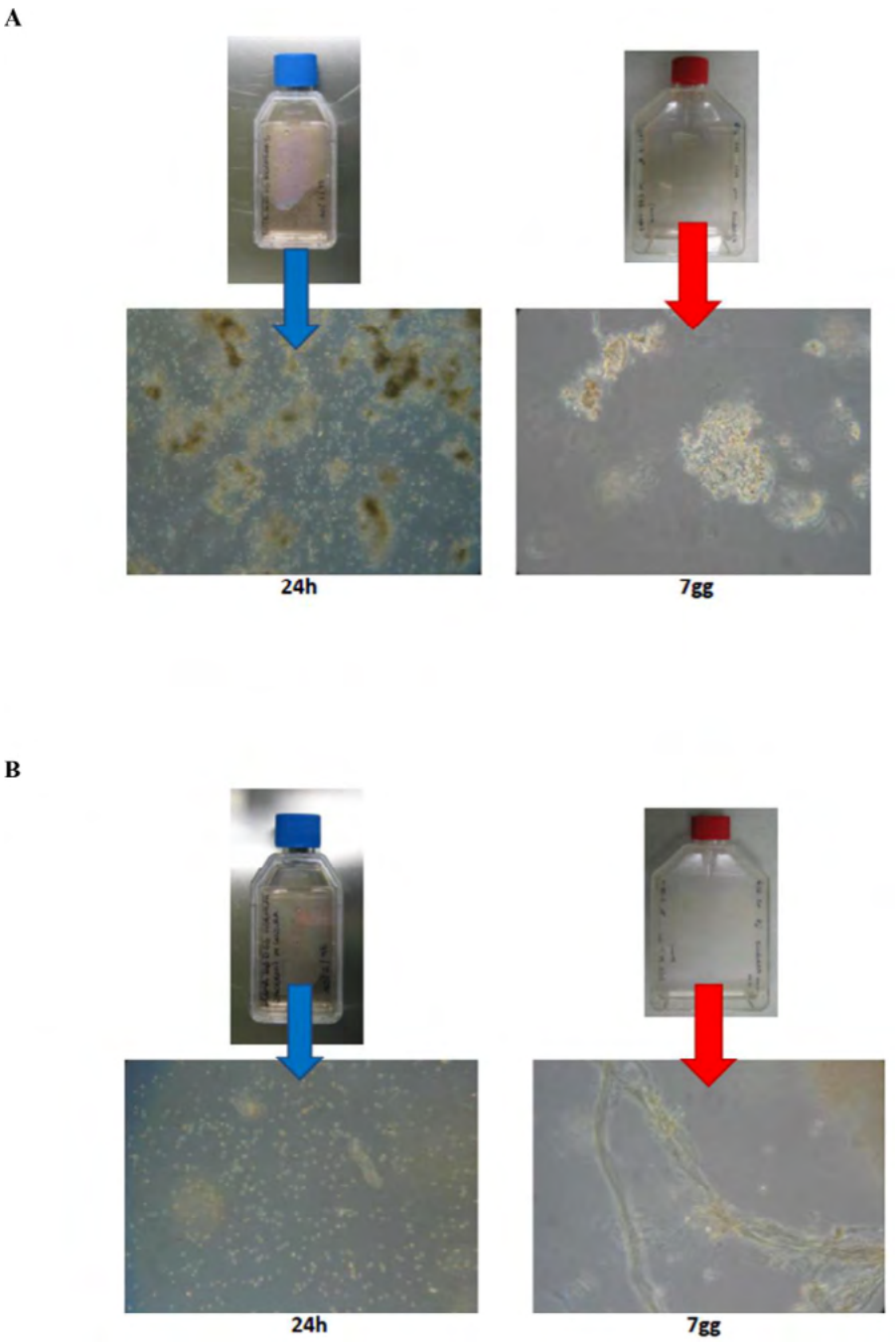
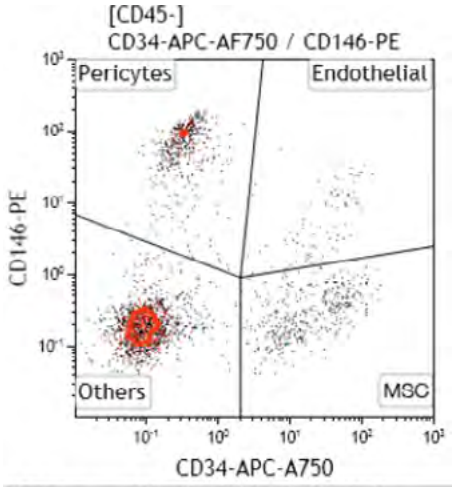


Figure 5. Morphological Analysis of Disaggregated Skin Tissue (A) and Ded/Dermis (B) after 24 hr and 7 Days of Culture. Morphological analysis was performed using light microscopy after 24 hr and 7 days of culture in the presence of RPMI medium on skin tissue and Ded/dermis tissues. Please click here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

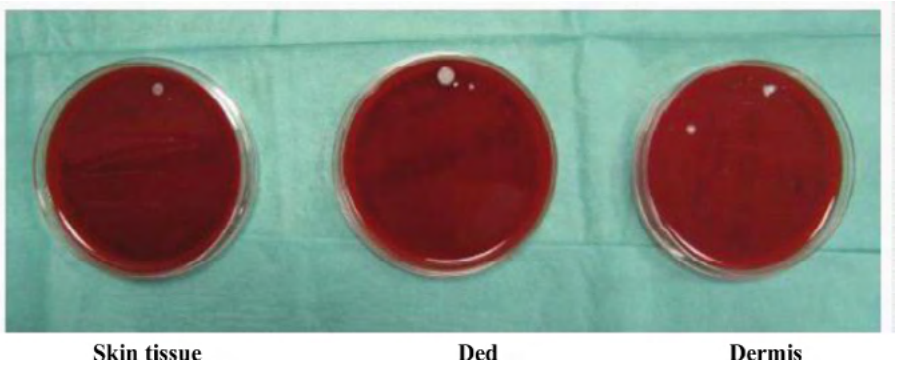


Figure 7. Microbiological Analysis of Disaggregated Skin Tissue, Ded and Dermis Samples. Small fragments of disaggregated tissue samples were seeded on Columbia agar added of 5% sheep blood (BioMerieux Company) under laminar flow hood and incubated at 37 °C for three days to perform the microbiological 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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11

ARTICLE

Dermoscopy in Female Androgenic Alopecia: Method Standardization and Diagnostic Criteria

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Original Article

Dermoscopy in Female Androgenic Alopecia: Method Standardization and Diagnostic Criteria

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ABSTRACT

Objective: Establishing the trichoscopy criteria of female androgenic alopecia (FAGA). **Design:** Trichoscopy images were retrospectively evaluated. **Setting:** Dermatologic hospital-based clinic and private practice offices. **Patients and Methods:** One hundred and thirty-one females (59 with androgenic alopecia, 33 with chronic telogen effluvium (CTE), 39 healthy controls). The diagnosis was based on clinical examination and confirmed by histopatology. **Main Outcome Measure:** Trichoscopy results obtained in frontal, occipital and both temporal areas of the scalp under a 20-fold and 70-fold magnification, including average hair thickness, number of 'yellow dots' and vellus hairs, number of hairs in one pilosebaceous unit and percentage of follicular ostia with perifollicullar hyperpigmentation. **Results:** Average hair thickness in frontal area versus occiput was, respectively, 0.061 ± 0.008 mm versus 0.058 ± 0.007 mm in healthy controls, 0.047 ± 0.007 mm versus 0.052 ± 0.008 mm in androgenic alopecia and 0.056 ± 0.007 mm versus 0.053 ± 0.009 mm in CTE. Mean percentage of thin hairs (< 0.03 mm) in androgenic alopecia was 20.9 ± 12% and was significantly higher than in healthy controls (6.15 ± 4.6%, $P < 0.001$) or in CTE (10.4 ± 3.9%, $P < 0.001$). The number of yellow dots, pilosebaceous units with only one hair and with perifollicular hyperpigmentation was significantly increased in androgenic alopecia. Classification and Regression Tree Analysis was performed to establish diagnostic criteria for FAGA. **Conclusion:** FAGA may be differentiated from CTE based on trichoscopy criteria. Major criteria are ratio of (1) more than four yellow dots in four images (70-fold magnification) in the frontal area, (2) lower average hair thickness in the frontal area compared to the occiput and (3) more than 10% of thin hairs (below 0.03 mm) in the frontal area. Minor criteria encompass increased frontal to occipital ratio of (1) single-hair pilosebaceous units, (2) vellus hairs and (3) perifollicular discoloration. Fulfillment of two major criteria or one major and two minor criteria allows to diagnose FAGA based on trichoscopy with a 98% specificity.

Key words: Alopecia, dermoscopy, hair, trichoscopy, videodermoscopy

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INTRODUCTION

Chronic hair loss frequently affects female patients, but there is little or no objective technology available to aid the dermatologist in setting a proper diagnosis and in monitoring treatment efficacy.[1] In particular, it may be difficult to differentiate between female androgenic alopecia (FAGA), a subtype of female pattern hair loss, and chronic telogen effluvium (CTE).[2]

Telogen effluvium is a self-limiting process and almost never causes obvious baldness,[3] whereas FAGA progresses in time, leading to a significant decrease in hair thickness, which over time may become cosmetically unacceptable and psychologically frustrating.[4]

Differences in natural history, prognosis and emerging new therapeutic possibilities[5] make differential diagnosis and early diagnosis of FAGA especially important. Currently, the diagnosis of FAGA is usually based on anamnesis and clinical findings, such as pattern of increased hair thinning, retention of the frontal hairline, and the presence of miniaturized hairs.[6,7] The semi-invasive technique of hair root analysis (trichogram) has a decreasing number of advocates among dermatologists. It is considered to be a poor indicator of FAGA and its severity.[8]

A scalp biopsy examination is usually performed to confirm the diagnosis of FAGA in clinically doubtful cases.[2,3] Histopathology findings in FAGA include a decrease in terminal/vellus hair ratio and decline in mean total follicle count with increasing grade of hair loss. This method also has limitations in everyday practice. Typically, punch biopsies are taken for vertical and horizontal embedding. These are 7-12 mm^2 in size and contain only a small number of hair follicles. Thus, some authors suggest performing multiple biopsies for representative sampling, which increases the invasiveness of this diagnostic technique and makes it even less useful for monitoring of treatment efficacy.[8]

Trichoscopy[9,10] is a newly developed method of hair image analysis, based on videodermoscopy of the hair and scalp. The method allows visualization of hair shafts at high magnification and performing measurements, such as hair shaft thickness, without the need of removing hair for diagnostic purposes. It also allows *in vivo* visualization of the epidermal portion of hair follicles and perifollicular epidermis.[11,12] Several reports raise the issue of potential usefulness of this technique in diagnosing hair and scalp disorders, such as microsporiasis,[13] androgenic alopecia,[14] alopecia areata,[15,16] lipedematous alopecia,[17] pediculosis[18] or inherited hair shaft abnormalities,[19,20] but the method has not been standardized yet and no criteria for diagnosing acquired diseases of hair and scalp have been established.

Objective

The aim of the study was to establish a standardized method of trichoscopy in acquired hair loss and to establish trichoscopy criteria for diagnosing FAGA.

Design

Trichoscopy images from female patients with FAGA, CTE and healthy volunteers collected in the years 2005-2007 were retrospectively evaluated.

Study selection

The diagnosis was established by clinical examination and histopathology. FAGA was clinically suspected in cases of frontal accentuation ("Christmas tree" pattern), diffuse central or vertex/frontal (male pattern) with sparing of the occiput.[6] The clinical diagnosis of CTE was based on diffuse form of scalp hair thinning longer than 6 months.[3]

All patients suspected for FAGA or CTE had three 4-mm punch biopsy specimens taken from the immediately adjacent skin on the mid scalp and all specimens were sectioned horizontally. The terminal to vellus hair ratio (T:V) at the midisthmus level was used to set the diagnosis. The ratio equal or lower than 4:1 was indicative for FAGA and equal or higher than 8:1 together with anagen: Telogen ratio lower than 8:1 was indicative for CTE.[4]

Setting

Dermatologic hospital-based clinic and private practice offices.

PATIENTS AND METHODS

After obtaining approval from the hospital's review board, a total of 131 female patients were included in the study, 59 with FAGA, 33 with CTE and 39 healthy volunteers.

The mean age was 36.2 (18-59) years in patients with FAGA, 32.2 (18-56) years in patients with CTE and 37.8 (19-58) years in healthy controls. The differences were statistically not significant.

Trichoscopy has been performed with the Fotofinder II videodermoscope, which permits scalp visualization at a 20-160-fold magnification. The device is equipped with software that allows to carry out measurements of structures visualized in magnified images and provides results in real scale. Images of the scalp were taken at a 20-fold magnification, which allows high-quality enlargement of 1 cm^2 of the scalp area to the size of a computer screen and at a 70-fold magnification, which magnifies in a similar manner an area of 9 mm^2.

In each patient, one image at a 20-fold magnification and four images at a 70-fold magnification were taken in each of the following four areas: Frontal, occipital, right temporal and left temporal.

Hair thickness was measured at a 70-fold magnification, in direct proximity to follicular orifices. Hairs have been identified as 'thin hairs' (below 0.03 mm), 'medium-size hairs' (0.03-0.05 mm) and 'thick hairs' (above 0.05 mm).

The images have been evaluated in accordance with the scheme presented in Table 1.

Statistical analysis was performed with the use of Student's *t*-test for paired samples and with analysis of variance (ANOVA). The Classification and Regression Tree technique was used to establish diagnostic criteria.

Table 1: Scheme of the trichoscopic images evaluation in the presented study

Parameter	Method of evaluation
Vellus hairs	Number of vellus hairs calculated in one field of vision (FOV) at 20-fold magnification
	Total number of vellus hairs in four FOVs at 70-fold magnification
	Highest result in one FOV from the above (at 70-fold magnification)
Distribution of hair thickness	Percentage of thin hairs (< 0.03 mm)
	Percentage of medium-sized hairs (0.03-0.05 mm)
	Percentage of thick hairs (above 0.05 mm)
	Mean hair thickness (mm)
Pilosebaceous units	Percentage of single-hair units (at 20-fold magnification)
	Percentage of double-hairs units
	Percentage of triple-hairs units
Yellow dots	Number of yellow dots per FOV calculated at 20-fold magnification
	Number of yellow dots per FOV calculated in four FOV at 70-fold magnification
	Highest result from the above (at 70-fold magnification)
Perifollicular yellow discoloration (hyperpigmentation)	Percentage of follicular ostia with perifollicular yellow discoloration calculated 20-fold magnification
	Number of follicular ostia with perifollicular yellow discoloration in four FOVs at 70-fold
	Highest result from the above (at 70-fold magnification)
Other	Number of white dots (scarified follicular ostia), cadaverized hairs, broken hairs
	Degree of desquamation (rated 0-4)
	Type of blood vessels
	Presence of exclamation mark hairs

RESULTS

Hair thickness

Both in healthy controls as well as in patients with CTE, the thickest hairs have been observed in the frontal area whereas the thinnest in the occipital area. In the healthy control group, the mean hair thickness was 0.061 ± 0.008 mm in the frontal area vs. 0.058 ± 0.007 mm in the occipital area ($P < 0.001$). In telogen effluvium, the values were 0.056 ± 0.007 mm vs. 0.053 ± 0.009 mm, respectively ($P < 0.001$). In FAGA, the smallest average thickness of hair roots has been observed in the frontal area, with 0.047 ± 0.007 mm compared to 0.052 ± 0.008mm in the occipital area ($P < 0.001$) [Figure 1].

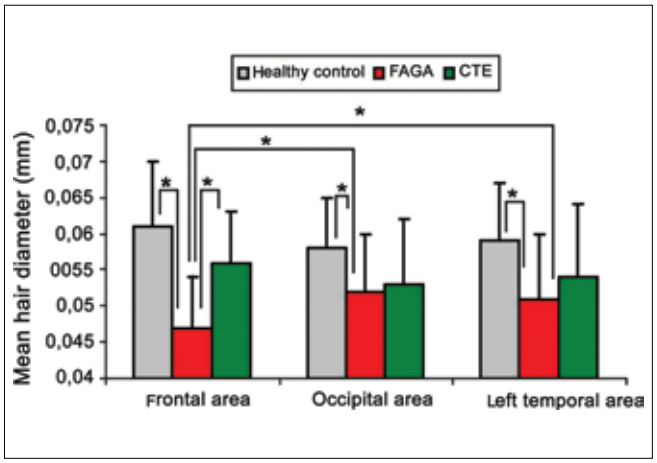

Figure 1: Mean hair diameter in frontal, occipital and left temporal areas of patients with female androgenic alopecia (FAGA), chronic telogen effluvium (CTE) and healthy controls. Asterix indicates the most important, statistically significant differences between them ($P < 0.001$)

Differences between the left and right temporal areas were statistically not significant in any of the investigated parameters. Thus, results for only one (left) temporal area are presented in figures.

In all assessed areas, the smallest average thickness of the hair roots has been noted in patients with FAGA [Figure 1]. Compared with healthy controls, hair thickness of patients with FAGA was significantly reduced in the frontal ($P < 0.001$), occipital ($P = 0.002$), left temporal ($P < 0.001$) and right temporal area ($P < 0.001$).

The largest average percentage of thin hairs (below 0.03 mm) was observed in FAGA in the frontal area (20.9 ± 12%) and it was significantly different compared to patients with telogen effluvium (10.4 ± 3.9%) and healthy volunteers (6.15 ± 4.6%, $P < 0.001$). An increase in the percentage of thin hairs was accompanied by an increase in the proportion of medium-sized hairs and a simultaneous decrease in the proportion of thick hairs. As the process occurred, the shift toward an increase of vellus hairs has also been observed in the occiput of FAGA patients.

The mean proportion of thin, medium-sized and thick hairs in the occipital area of FAGA patients was 14.2 ± 8.9%, 31.5 ± 15.8% and 54 ± 4.9%, respectively. In the control group, the respective proportions were 6 ± 5.1%, 20.6 ± 13.4% and 73.4 ± 5.8%. In telogen effluvium, the respective values were 11.9 ± 3.9%, 29.7 ± 15.9% and 58.6 ± 8.5% [Figure 2].

Image analysis showed that thin hairs (hairs below 0.03 mm) differ in CTE from FAGA. In CTE thin hairs are short, sharp ended, and become gradually thinner from their proximal to

distal end. They are newly regrowing hairs. [Figure 3a and b]. In FAGA thin hairs correspond to vellus hairs resulting from progressive hair follicle miniaturization. They are evenly thin and bluntly ended [Figure 3c and d]. A number of five or more short and sharp-ended hairs in four fields of vision at a 70-fold magnification in both the frontal and the occipital or temporal area was found in 18/33 (54.5%) patients with CTE and in none of the patients with FAGA.

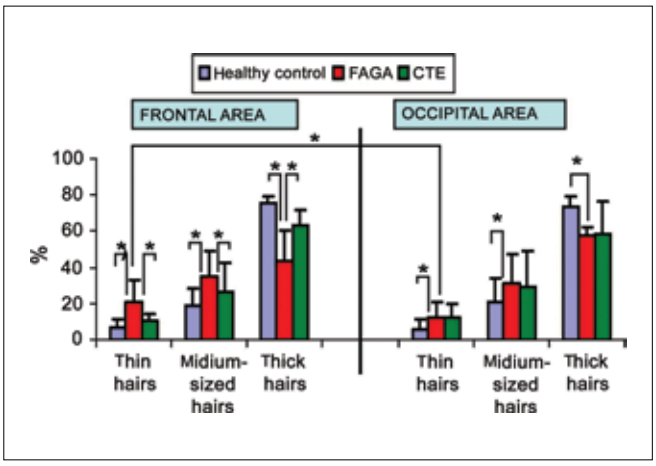

Figure 2: Percentage of thin, medium-sized and thick hairs in frontal and occipital areas in healthy controls, patients with female androgenic alopecia (FAGA) and chronic telogen effluvium (CTE). Asterix marks statistically significant differences ($P < 0.05$)

Pilosebaceous units

Hairs usually are present in groups of few hair roots growing from one follicular orifice. The number of hairs in pilosebaceous units has been evaluated by trichoscopy at the 20-fold magnification. The percentage of single-hair, double-hair and triple-hair units was evaluated.

In healthy controls and in CTE, the highest proportion of single-hair pilosebaceous units was observed in the temporal areas. The mean percentage of single-hair units in this area was 42 ± 12% in CTE and 32 ± 15% in healthy controls.

In patients with FAGA, the mean percentage of single-hair pilosebaceous units was highest in the frontal area

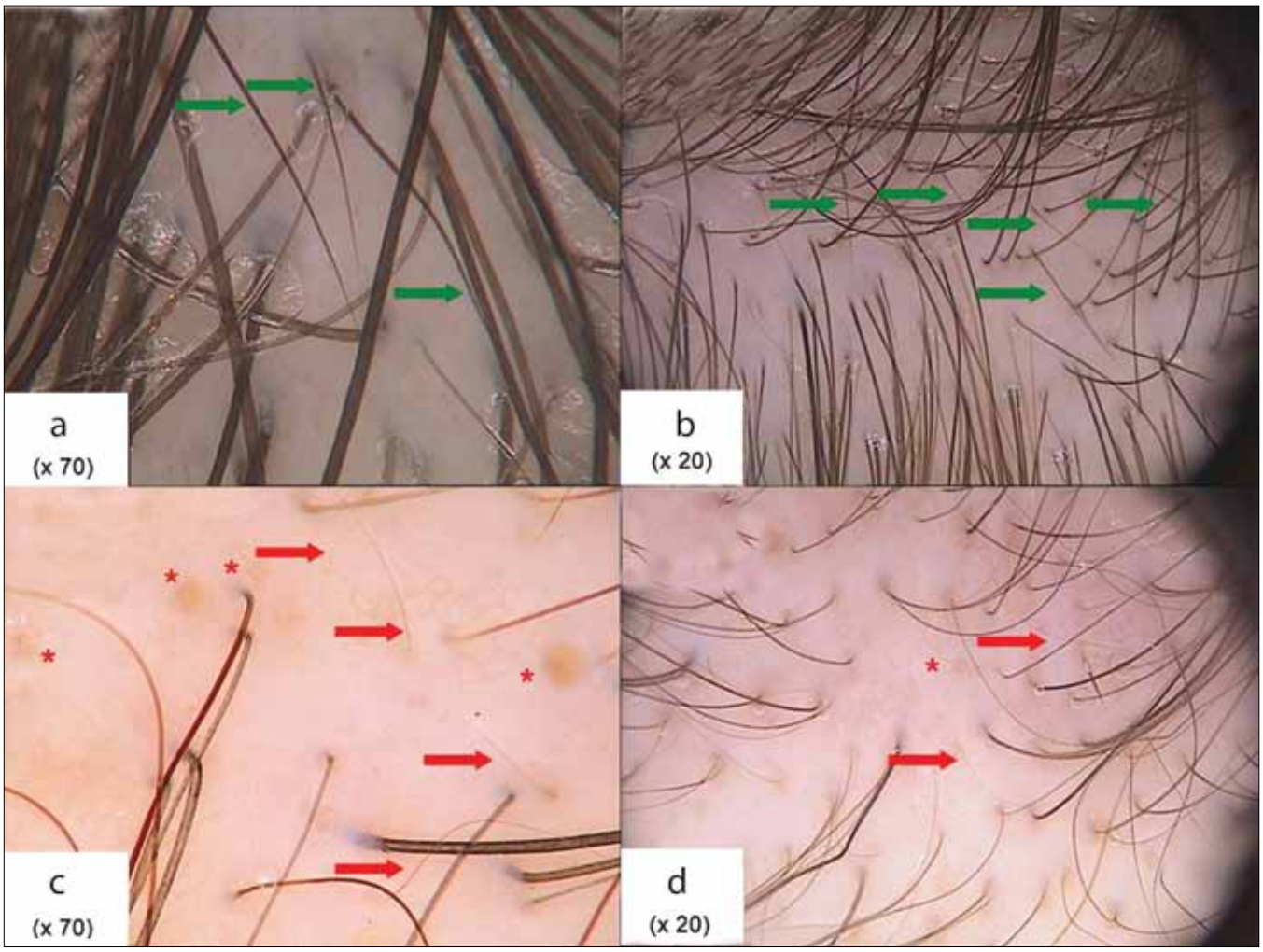

Figure 3: Trichoscopy of frontal scalp area in androgenic alopecia (a and b) and chronic telogen effluvium (c and d). Images presented in 70-fold (a and c) and 20-fold magnification (b and d). Red arrows: Vellus hairs; green arrows: Short, new anagen hairs; red asterix: Yellow dots

(65.2 ± 19.9%). This was significantly more than in telogen effluvium (39.0 ± 13.4%, $P < 0.001$) and in healthy controls (27.3 ± 13%, $P < 0.001$). The smallest difference between these groups in the proportion of single-hair pilosebaceous units has been noted in the occipital region. The numbers for healthy controls, FAGA and telogen effluvium were 22.6 ± 12.6%, 36.8 ± 18.6% and 31 ± 23%, respectively [Figure 4]. The difference between FAGA and healthy control was statistically significant at $P < 0.001$. The mean proportion of single-hair units in the frontal area to occipital area was 2.56 in healthy controls, 3.4 in telogen effluvium ($P = 0.01$) and 10.4 in FAGA ($P < 0.001$).

Yellow dots

Yellow dots were evaluated at a 20-fold magnification and at a 70-fold magnification. Results are given as total number of yellow dots in one field of vision (FOV) at the 20-fold magnification and as total number seen in four FOVs at the 70-fold magnification. In both calculation methods, the number of yellow dots was significantly higher in patients with FAGA as compared with healthy controls or with patients with CTE. However, in general, the number of yellow dots per 1 mc^2 was on average 20% higher when evaluated at the 70-fold magnification, as compared with the 20-fold magnification. This difference resulted from better visualization of small trichoscopy structures at higher magnifications. Figure 5 shows the mean numbers of yellow dots in healthy controls, FAGA and telogen effluvium when analyzed in four fields of vision at a 70-fold magnification. The highest number of yellow dots in patients with FAGA was noted in the frontal area (8.86 ± 4.8/4 fields of vision at the 70-fold magnification). The corresponding number in the occipital area was 1.59 ± 2.0.

PERIFOLLICULAR DISCOLORATION

The percentage of hair-containing units with perifollicular discoloration was evaluated at the 20-fold magnification and in four FOVs at the 70-fold magnification. Both methods yielded highly comparable results. Thus, results are presented for the 20-fold magnification only.

Perifollicular discoloration was found significantly more often in FAGA as compared with healthy controls or patients with CTE. The mean percentage of hair follicles with surrounding discoloration in FAGA was 32.4 ± 4.7% in the frontal area and 6.6 ± 2% in the occipital area ($P < 0.001$).

OTHER PARAMETERS

Other parameters (white dots, cadaverized hairs, broken hairs, degree of desquamation, predominant type of blood vessels, presence of exclamation mark hairs) were evaluated in accordance with the scheme presented in Table 1 and gave results that were non-significant for setting up criteria for the diagnosis of FAGA.

CLASIFICATION AND REGRESSION TREE

Data obtained in this study were used to define diagnostic trichoscopy criteria for FAGA by employing a Classification and Regression Tree analysis. These allowed to define three major and three minor criteria into an algorithm, which gave a 98% method specificity for FAGA.

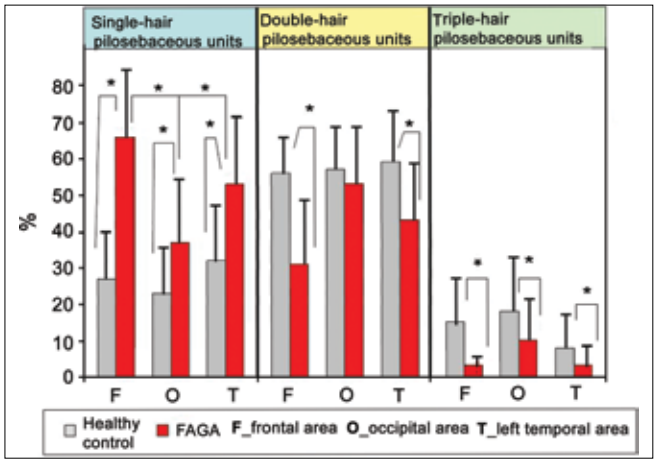

Figure 4: Percentage distribution of pilosebaceous units with one, two and three hairs. In chronic telogen effluvium patients as well as in the healthy controls, the distribution was similar. Thus, only healthy control results and patients with androgenic alopecia are presented

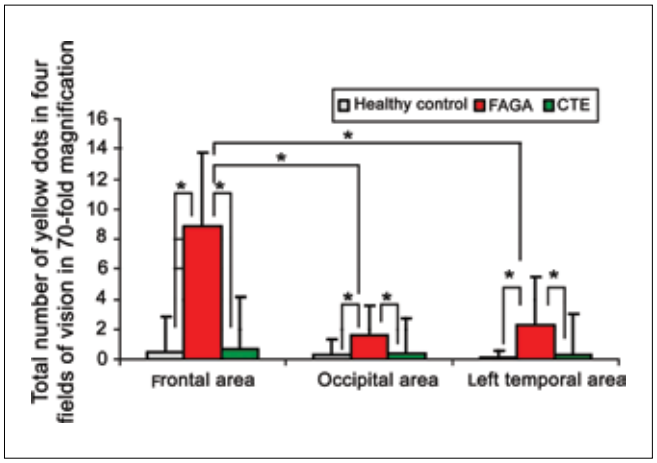

Figure 5: Yellow dots in frontal, occipital and left temporal areas of all three groups of patients, presented as a number counted in four field of visions at 70-fold magnifications. Asterix marks statistically significant differences ($P < 0.001$)

Major criteria

1. More than four yellow dots in four images at a 70-fold magnification in the frontal area.
2. Lower average hair thickness in the frontal area in comparison with the occiput (calculated from not less than 50 hairs from each area).
3. More the 10% of thin hairs (below 0.03 mm) in the frontal area.

Minor criteria

1. Ratio of single-hair unit percentage, frontal area to occiput >2:1
2. Ratio of number of vellus hairs, frontal area to occiput >1.5:1
3. Ratio of hair follicles with perifollicular discoloration, frontal area to occiput >3:1.

Fulfillment of two major criteria or one major and two minor criteria is required to diagnose FAGA based on trichoscopy.

In order to confirm this diagnostic model, a linear regression analysis was performed in which the dependent variable was the diagnosis of FAGA and the diagnostic criteria were independent variables. An analytical model, which was constructed in this manner showed a potential diagnostic sensitivity of this method at the level of 72%.

TRICHOSCOPY REPORT

Based on results collected in this study, we established a trichoscopy report form that contains most important trichoscopy findings in diffuse hair loss and diagnostic criteria for FAGA [Table 2]. Elements, which were not found to be of diagnostic value in this or previous studies,[11,12] were not included in this trichoscopy report form. These include trichoscopy results from parietal regions and results from magnifications that were less precise or significantly less comfortable to handle.

DISCUSSION AND CONCLUSIONS

FAGA, a disease in the spectrum of female-pattern hair loss, is characterized by progressive miniaturization of hair follicles, mediated most probably by dihydrotestosterone within the follicle, and may affect women with normal levels of circulating androgens.[21] The diagnosis is usually based on anamnesis and clinical findings, such as early age of onset, the pattern of increased hair thinning

Table 2: Trichoscopy report scheme

	Calculations in frontal and occipital areas	FAGA criteria
Major criteria	Total number of yellow dots in four fields of vision*	>4 in frontal area
	Mean hair thickness (mm)*	Lower in frontal area
	Percentage of hairs*	More than 10% thin hairs in the frontal area
	Thin hairs (<0.03 mm)	
	Medium-sized hairs (0.03-0.05 mm)	
	Thick hairs (>0.05 mm)	
Minor criteria	Percentage of units in one field of vision**	Ratio of single-hair units, frontal area to occiput > 2:1
	Single-hair unit	
	Two-hair units	
	Three-hair units	
	Total number of vellus hairs in four fields of vision*	Ratio of frontal area to occiput > 1.5:1
	Percentage of hair follicles with perifollicular discoloration**	Ratio of frontal area to occiput > 3:1
	Other observations (i.e., exclamation hairs, cadaverized hairs, white dots)	

*70-fold magnification, **20-fold magnification

over the frontal/parietal scalp with greater hair density over the occipital scalp, retention of the frontal hairline and the presence of miniaturized hairs.[6,7,22] Histologically, the disease is characterized by miniaturization of a proportion of follicles and an increased percentage of hair in telogen in the affected area.[7] In advanced stages of disease, when these features are obvious, the diagnosis is not problematic. However, in early disease and in patients in whom other causes of hair loss coexist, the diagnosis may be challenging.

In this study, trichoscopy criteria were established, which allow to diagnose FAGA with 98% specificity. These criteria are based on findings that have partly been known from clinical observations and other diagnostic methods but could not be quantified before trichoscopy was developed. These findings relate to predominance of hair miniaturization in the frontal area compared with the occiput.

This clinical and histopathological observation has been identified and quantified by trichoscopy as lower average hair thickness in the frontal area in comparison with the occiput, more the 10% of thin hairs (below 0.03 mm) in the frontal area and ratio of vellus hair number (frontal area to occiput) above 1.5:1.

A novel approach to quantify hair density in practical dermatology is evaluation of the number of hairs in

one pilosebaceous unit. This was previously not possible with the classical diagnostic method or the recently developed phototrichogram.[23] The number of hairs in one pilosebaceous unit varies from one to three in healthy persons. Occasionally, a four-hair unit may be found. Our results show that the number of single-hair pilosebaceous units is significantly increased in the frontal area in patients with FAGA compared with the occiput (a ratio above 2:1). Hair thinning, decreased number of hairs in pilosebaceous units and predominant prevalence in the frontal area are main features distinguishing FAGA from most other hair diseases, especially alopecia areata.[11]

Clinical 'patterning' in patients with female-pattern hair loss may be with frontal accentuation ("Christmas tree" pattern), diffuse central or vertex/frontal (male pattern) with sparing of the occiput.[6] Recently, studies have demonstrated that in patients with FAGA, changes observed in the occiput are similar to those in the frontal area, but are less pronounced. Using the phototrichogram method, both Ekmekci[24] and van Neste[1] showed decreased hair density and an increased percentage of thin hair roots (< 0.04 mm) in the occipital area of FAGA patients compared with healthy controls. Our results also confirm the occurrence of changes typical for androgenic alopecia in other areas than those considered to be 'androgen-dependent.' We have shown that in the occipital area of patients with FAGA, the average hair diameter is significantly decreased, the percentage of vellus hairs is increased and the number of single-hair pilosebaceous units is higher than in the occipital area of healthy controls. Also, other features of FAGA, such as yellow dots and perifollicular discoloration, were found in the occipital area of patients with FAGA but not in healthy controls. Assessment of these trichoscopy features in temporal areas gave intermediate values between results obtained for the frontal region and the occiput in both FAGA and CTE. It may be concluded that trichoscopy of temporal areas may be ignored in dermatological practice. Interestingly, it has been observed in healthy controls that the temporal areas have the highest number of vellus hairs and single-hair pilosebaceous units, indicating that physiologically these areas have the lowest hair density.

A major trichoscopy criterion of FAGA is the presence of yellow dots, which reflect hair follicle ostia lacking any hairs (empty hair follicles). It may be suggested that yellow dots in FAGA result from the presence of sebaceous lobules, which in histopathology appear large in relation to the miniaturized follicles.[25] We hypothesize that these sebaceous glands are still active after advanced hair follicle miniaturization and produce sebum, which creates intra-epidermal sebum lagoons. These sebum lagoons appear as yellow dots in trichoscopy.

Yellow dots have been previously described in alopecia areata[11,12] and have been recently suggested as indicative of alopecia areata incognita (Kligman's telogen effluvium) in patients with diffuse hair loss.[17] Our results show that yellow dots may be characteristic for a wide spectrum of hair diseases and they may also represent a wider than previously anticipated spectrum of histopathological appearances of follicle ostia and infundibula. Our results show that yellow dots are one of the most important trichoscopy features distinguishing FAGA from CTE.

Another important trichoscopy finding is perifollicular discoloration of the skin. This feature, called by some authors as 'hyperpigmentation' or 'peripilar sign' reflects the presence of perifollicular lymphocytic infiltrates in early androgenic alopecia.[26] Our results confirm the presence of perifollicular discoloration in FAGA, although we found that in FAGA the percentage of hair follicles with this abnormality is significantly higher in the frontal area compared with the occiput. According to statistical analysis, a ratio of hair follicles with perifollicular discoloration and frontal area to occiput higher than 3:1 is highly indicative of FAGA.

CTE has no specific trichoscopy features apart from an increased proportion of short, sharp-ended hairs. A number of five or more in four fields of vision at a 70-fold magnification in both the frontal and another (occipital or temporal) was found highly indicative of CTE. Most likely, these are regrowing hairs in early anagen stage and their increased number indicates an accelerated hair cycle, resulting in an intensive replacement of hair roots. However, CTE may rather be a diagnosis made by exclusion of other causes of hair loss, than by direct fulfillment of specific trichoscopy criteria.

In conclusion, the results of our study indicate that FAGA may be differentiated from CTE and the diagnosis of FAGA may be established based solely on trichoscopy criteria.

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ARTICLE

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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ARTICLE

Advances in Stem Cell-Based Therapy for Hair Loss

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Advances in Stem Cell-Based Therapy for Hair Loss

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Abstract

OBJECTIVE: Hair loss is a quite common condition observed in both men and women. Pattern hair loss also known as androgenetic alopecia is the most common form of hair loss that is thought to affect up to 80% of Caucasian men and up to 40% of Caucasian women by age of 70, and it can have quite devastating consequences on one's well-being, including lower self-esteem, depression and lower quality of life. To date there have only been 2 FDA approved medications, minoxidil and finasteride, but their effects are often unsatisfactory and temporary, in addition to having various adverse effects. Stem cell-based therapies have recently received lots of attention as potential novel treatments that focus on reactivating hair follicle stem cells and in this way enhance hair follicle growth, regeneration and development. Stem cell-based therapy approaches include stem cell transplant, stem cell-derived conditioned medium and stem cell-derived exosomes.

MATERIALS AND METHODS: A combination of following key words was utilized for a PubMed search: cell-based therapy, hair loss, alopecia, hair regrowth; abstracts were screened and included based on the content relevant to hair loss and stem-cell based therapy.

RESULTS: Preclinical research utilizing these approaches has blossomed in the past decade along with a more limited number of clinical studies, overall demonstrating very promising findings.

CONCLUSION: However, stem cell-based therapies for hair loss are still at their infancy and more robust clinical studies are needed to better evaluate their mechanisms of action, efficacy, safety, benefits and limitations. In this review, we provide the resources to the latest preclinical studies and a more detailed description of the latest clinical studies concerning stem cell-based therapies in hair loss.

Keywords

hair loss; alopecia; stem cell-based therapy; transplant; conditioned medium; exosome; hair regrowth; hair regeneration

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Conflicts of Interest: The authors declare that they have no conflict of interest to disclose.

Introduction:

Hair loss, particularly, pattern hair loss (PHL) as its most common form, occurs quite commonly in both women and men, and often leads to a significant decrease in quality of life[1]. It is believed that over 80% of Caucasian men and up to 42% of Caucasian women at the age of 70 are affected by male pattern hair loss/androgenetic alopecia (MPHL/AGA) and female pattern hair loss (FPHL), respectively[1]. Hair is considered a major feature of beauty and esthetic appearance; hence hair loss has a major impact on one's self-perception, self-esteem, and can lead to depression and other mood disorders[2]. Furthermore, some postulate early onset of AGA to be associated with a heightened risk of development of myocardial infarction and metabolic syndrome[3].

It is thought that the Wnt- β-catenin pathway plays a major role in pathogenesis of hair loss.[4] To date, there are only 2 FDA approved medications for treatment of hair loss, minoxidil (a vasodilator) and finasteride (a selective inhibitor of the type II and III isoforms of 5α-reductase). However, these medications have been far from perfect; both have been associated with limited efficacy, duration of effect, and several important side effects[5, 6].

PHL is a form of non-scarring alopecia. PHL is characterized by defects in and loss of hair progenitor cells, while hair follicle stem cells (HFSCs) remain viable. This notion in particular makes PHL a reversible condition[7, 8], and current and novel treatment modalities attempt to utilize the existent viability and responsiveness of HFSCs as to reverse hair loss pathology and promote hair growth. Providing adequate signals and environment to reactivate HFSCs and regrow a hair follicle is of particular interest to the hair regeneration scientific and clinical community. In the past decade, hair regeneration research has plummeted, including the discoveries regarding stem-cell based therapies leading to many preclinical and some clinical studies with encouraging outcomes. Stem-cell transplant, stem cell-derived conditioned medium (CM) and stem cell-derived exosomes have recently gained a lot of attention as potential new agents to modify and enhance the signaling pathways that could induce HFSC reactivation, hair cycle and hair follicle regeneration. In this review, we will provide resources to the preclinical studies, but our major focus will be on the latest clinical research as it relates to stem-cell based therapies, hair loss, and hair regeneration potential.

Discussion:

As briefly mentioned previously, stem-cell based therapies include three distinct prospective mechanisms: transplantation of multipotent stem cells from different sources, application of stem cell-derived CM and application of stem-cell derived exosomes[9]. Herein, we will address each of them individually by discussing current clinical studies, their results, respective benefits and limitations.

Transplantation of multipotent stem cells has become a well-accepted treatment option for hair loss (especially AGA). The sources of multipotent stem cells with regenerative potentials of hair follicles in the skin include adipose tissue[10], bone marrow[11], hair follicles from unaffected areas[12], and umbilical cord blood[13].

Owczarczyk-Saczonek et al[8] provide a thorough review of pre-clinical discoveries of promising results and benefits of stem-cell based transplant therapies. Results of clinical studies are further discussed below.

Elmaadawi et al[14] studied the safety and efficacy of the autologous bone marrow-derived mononuclear cells (BMMCs) including stem cells in comparison to follicular stem cells (FSCs) obtained from the unaffected scalp areas in 20 patients with alopecia areata (AA) and 20 patients with AGA. All patients underwent one treatment session with autologous stem cells (BMMCs or FSCs) that were injected intradermally. Evaluation by immunostaining and digital dermoscopy 6 months post-treatment demonstrated significant improvement of both conditions with no significant difference between treatment groups and no adverse events.

Rigenera® is a technology that obtains autologous mature stem cells from biopsies of a patient using a preparation system for mechanical disintegration and filtering of solid tissues. In a study the cell suspension was injected into the scalp of 11 patients affected by AGA. 23 weeks post-treatment there was a 29%±5% increase in hair density in the scalp area receiving treatment as compared to the area receiving placebo[15]. Gentile et al[15] suggested that bulge-derived HFSCs can be isolated with this newly discovered method to avoid the challenges concerning cell culturing and more importantly that they have the ability to enhance hair density in patients with AGA.

Multipotent stem cells arising from the adipose tissue – the adipose-derived stromal vascular cells (ADSVCs) or adipose-derived regenerative cells (ADRCs) refer to the stromal vascular fraction-derived freshly used primary multipotent stem cells. When these cells are cultured, they attain additional features and become a population of mesenchymal stem cells (MSCs) which are referred to as adipose-derived stem cells (ADSCs)[16, 17]. Anderi et al[16] studied ADSVCs in a total of 20 patients suffering from AA[16]. There was a statistically significant improvement of hair thickness especially 6 months post-treatment. Only 1 out of 20 patients did not demonstrate any increase in hair diameter. Furthermore, there was a statistically significant increase in hair density 3 and 6 months post-treatment; 18 out of 20 patients demonstrated improvement while only 2 out of 20 patients did not show any increase in hair density. Lastly, there was also a statistically significant decrease in hair-pull test results 3 and 6 months post-treatment; only 2 out of 20 patients did not demonstrate any decrease in hair-pull test scores. Anderi et al[16] suggested autologous ADSVCs graft to be safe and effective treatment modality for AA.

Zanzottera et al[10] utilized the Rigenera® device to prepare autologous ADMSCs obtained during hair transplant procedure. The suspension was then applied to the scalp areas undergoing hair transplant in 3 patients suffering from AGA. Monthly follow up revealed a more rapid healing of transplant-induced wounds. Furthermore, there was a continuous improvement in hair growth and a shorter telogen phase two months post-treatment.

Another study found benefit of primary pluripotent ADRCs in enhancing hair growth. Particularly, addition of stromal vascular fraction-derived stem cells to the adipose tissue in a transplant procedure involving 6 patients suffering from male or female PHL demonstrated a

statistically significant 23% increase in mean hair count compared to 7.5% increase in patients treated with adipose tissue alone[17].

The dermal papilla (DP) region is an important area of the hair follicle that contains MSCs which participate in inducing hair growth and controlling hair cycle. DP cells are surrounded by dermal sheath cup (DSC) cells which are essential for DP cell regeneration and proliferation and therefore hair growth, as well[18]. It is proposed that circulating androgens deregulate DP cell-derived signaling leading to inhibition of canonical Wnt-β-catenin pathway and hair loss in AGA_4. Besides DP cells, the multipotent stem cells from the bulge region are also thought to depend on DSC cells[19]. In a study by Tsuboi et al[20], 50 male and 15 female patients received a single injection treatment of autologous DSC cells at concentrations 7.5×10^6, 1.5×10^6, or 3.0×10^5 DSC cells or a placebo in 4 randomized distinctive scalp regions and were followed-up at 3, 6, 9 and 12 months post-treatment. There was a significant increase in total hair density and cumulative hair diameter at the 3.0×10^5 DSC cell injection location 6 and 9 months post-treatment. These results suggested that autologous DSC cell injection at minimal concentration is a potential safe and useful additional modality for treatment of PHL in both males and females.

A new focus is being placed on stem-cell secreted bioactive molecules such as growth factors, cytokines, chemokines, and others, as potential key regulators of hair follicle cycle and regeneration[9]. Particularly, it is thought that up to 80% of regenerative properties of transplanted stem cells come from paracrine factor signaling[21, 22]. Stem cells secrete such factors including nucleic acids, extracellular vesicles (exosomes included) and proteins, thus inducing paracrine signaling[23, 24]. These factors are components of a secretome. In other words, secretome represents a set of signaling molecules including nucleic acids, extracellular vesicles, and proteins secreted by stem cells. When a cultured stem cell-derived secretome is present in a nutrient-rich medium it is referred to as a stem cell-derived "conditioned medium" (CM)[25].

Several studies focusing specifically on the ability of the extracellular matrix (EM) to induce hair regeneration are available and overall their results are promising[9]. Moreover, in comparison to other modalities, stem cell-derived CM provides additional benefits. For instance, the donor-recipient match that is normally required in a cell-based type of treatment is surpassed with CM because it represents a cell-free medium[26]. Additionally, there appears to be less risk of tumor development as well as benefits of easier preparation and lower cost[27, 28]. Although stem cell-derived CM-based therapy is at its early beginnings, many preclinical[9] and several clinical studies have shown encouraging results. The clinical studies will be discussed below.

Fukuoka et al[29] evaluated efficacy and safety of ADSC-CM in 25 patients (12 women and 13 men) diagnosed with female or male PHL; 1 male patient received a diagnosis of both AGA and AA. In this study, ADSCs were pretreated under hypoxic conditions that were previously shown to have the ability to induce secretion of various growth factors and cytokines with potential benefits for hair regrowth as compared to normoxic ADSCs[29, 30]. The ADSC-derived secretome is composed of hepatocyte growth factor, vascular endothelial growth factor, keratinocyte growth factor and platelet-derived growth factor[29]. This medium

was applied every 3–5 weeks by utilizing nappage and papule injection methods. All patients demonstrated a statically significant improvement in hair growth; 4 treatment sessions over a 3–4-month-period resulted in best results[29].

In another study of the same group on 22 patients (11 men and 11 women) with alopecia received ADSC-CM injections every 3–5 weeks for a total of 6 sessions. 10 patients (8 men and 2 women) were also part of a half-side comparison study. Trichogram evaluations before and after treatment demonstrated a statistically significant increase in hair numbers in both genders. In the half-side comparison study, the side receiving treatment exhibited a significant increase in hair numbers compared to the side of placebo[31]. Adverse events included post-procedural pain which negatively affected patient compliance.

ADSC-CM was also evaluated in 27 female patients suffering from FPHL. This group utilized a microneedle roller to apply ADSC-CM weekly for 12 consecutive weeks. Phototrichographic analysis revealed a statistically significant increase in both hair density and hair thickness, and no adverse events (including pain)[32].

Narita et al[33] evaluated efficacy of ADSC-CM in a total of 40 patients (21 men and 19 women) diagnosed with alopecia[33]. Patients underwent ADSC-CM intradermal injections monthly for a total of 6 months and had follow-up evaluations before and at 2, 4 and 6 months post-treatment. There was a significant increase in hair density and anagen hair rate in this study, as well as, dermal echogenicity and dermal thickness of the treated scalp.

Undoubtedly, CM demonstrates potential as a future hair regrowth therapy; however, like any treatment modality it poses certain limitations. Particularly, the type and level of factors present in a stem cell-derived CM can be highly variable, and standardization of its preparation will be of utmost importance to improve its clinical use and results[22]. Additionally, fast turnover and depletion of CM factors *in vivo* may necessitate large quantities and frequent application[34, 35]. We will now briefly discuss one particular component of CM that is considered an additional alternative stem-cell based therapeutic approach: the exosome.

Exosomes are extracellular vesicles of the smallest size, that act as cell-to-cell transporters and messengers by carrying signaling molecules including transcription factors, cytokines, and RNA[22, 36, 37]. Exosomes have been demonstrated as important modulators of paracrine signaling, and particularly, DP cell-derived exosomes could be of major importance for hair follicle regeneration[38]. Many of the preclinical studies show favorable outcomes; however, there are currently no clinical studies employing extracellular vesicle or exosome therapy for hair growth[9]. More preclinical and new clinical studies are needed to further characterize exosomes as a novel regenerative treatment for hair loss.

More robust studies are encouraged for the other two stem cell-based therapy approaches: the stem cell-based transplant and the stem cell-derived CM; several clinical trials currently are underway (ClinicalTrials.gov Identifiers: NCT01673789, NCT02865421, NCT03078686, NCT02849470, NCT03676400, NCT03662854, NCT 01501617). Several aforementioned studies have been completed and are awaiting results.

Conclusion:

Novel discoveries revolving around stem-cell based therapies provide encouraging steps towards developing more effective and successful hair loss treatments. Although these initial steps towards such discoveries are hopeful, there is still a limited amount of clinical data to fully support stem-cell based therapies. While stem-cell transplant, CM and exosome therapies demonstrate preclinical and some clinical success, each one of them has its own limitations that will need to be overcome. Stem cell transplant is a costly procedure, and it also raises concerns for tumorigenicity[24]. While CM and exosomes may be more affordable[26] and safe in terms of tumor development[27, 28] they both pose some problems. The cell-free nature of CM provides a safer and more immunocompatible environment, but makes isolating a composition-consistent CM challenging[26]. Similarly, there are currently no standard effective isolation methods for exosomes[39]. While results are certainly hopeful, larger and more robust double blind controlled clinical trials are needed to further assess the exact mechanisms, therapeutic potential and safety of stem-cell based approaches to hair loss management.

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14

ARTICLE

Adipose Derived Stem Cells and Growth Factors Applied on Hair Transplantation

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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 aim 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 of the main vascular network for the engraftment of transplanted hair and the healing process.

A number of researches underlined 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** indicats 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.

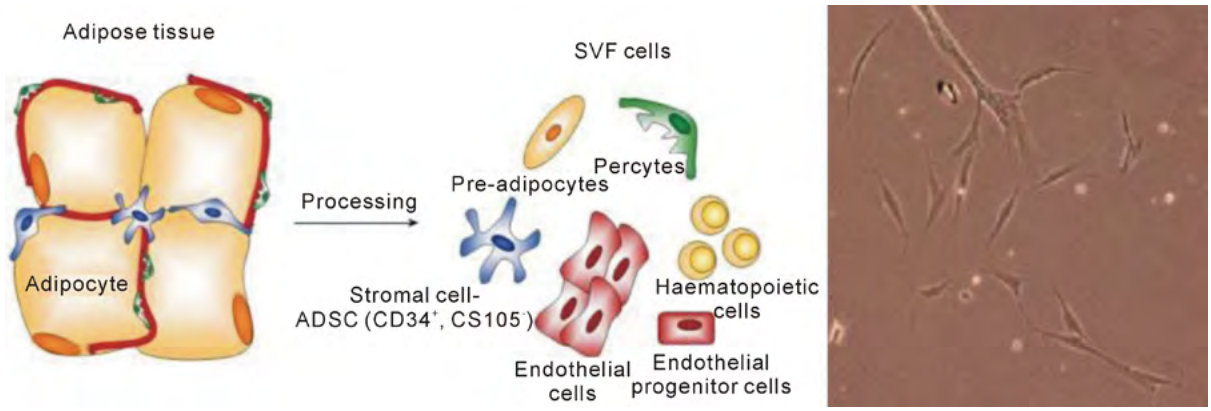


Figure 1. Adipose tissue and photo of ADCS by optical microscope.

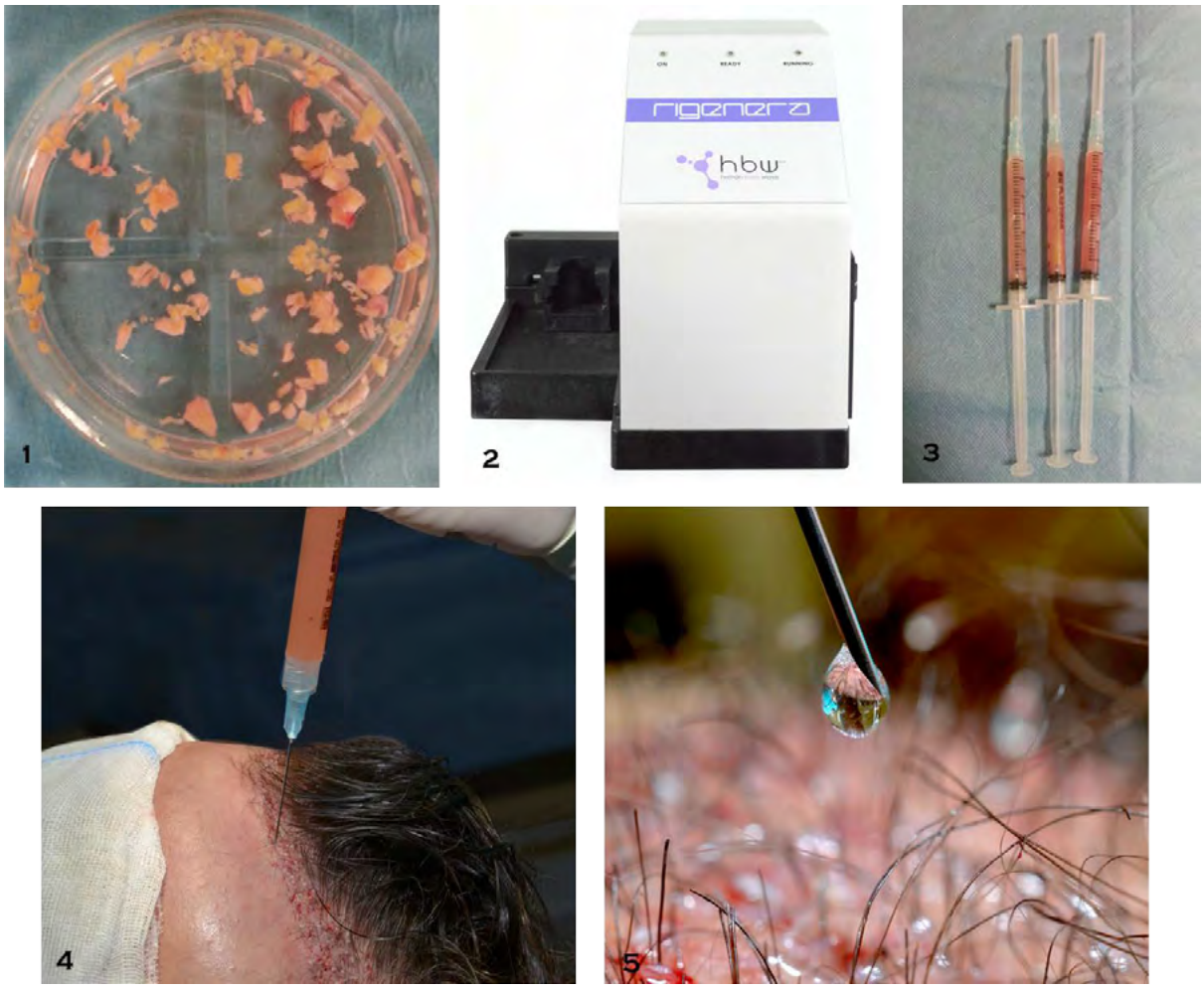


Figure 2. Rigenera System: 1) Hypo-derma and adipose tissue from the occipital region; 2) Rigenera system: a piece of tissue +1 ml of saline solution was inserted in Rigeneracons (CE/EC disposable medical devices containing a stainless steel grid with 100 hexagonal holes of 50 μ each surrounded by six 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.

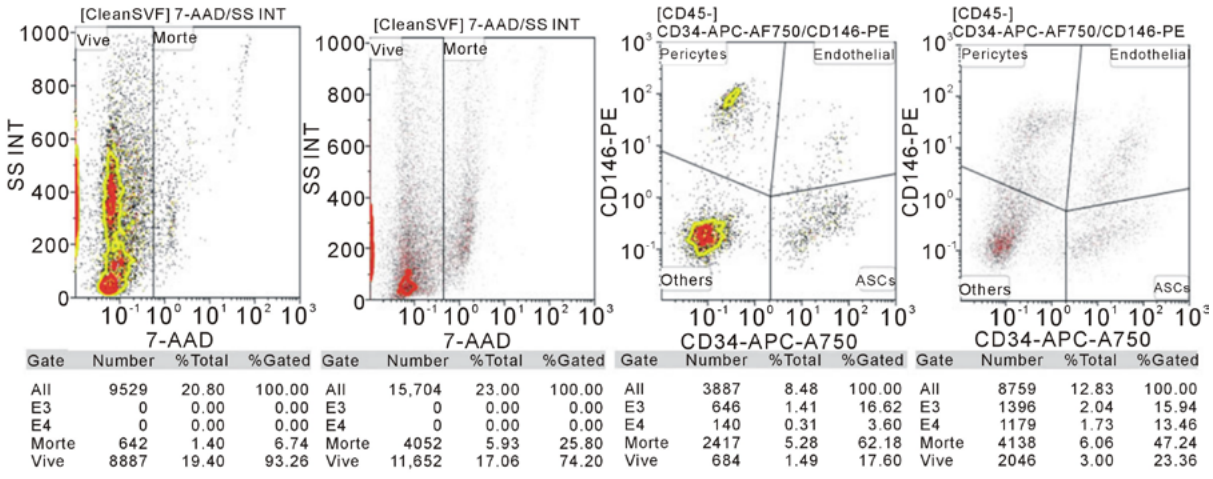
5. Results

5.1. Cell Suspension Analysis

The cellular vitality is 93% for the 1th patients and 74% for the 2th. 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).

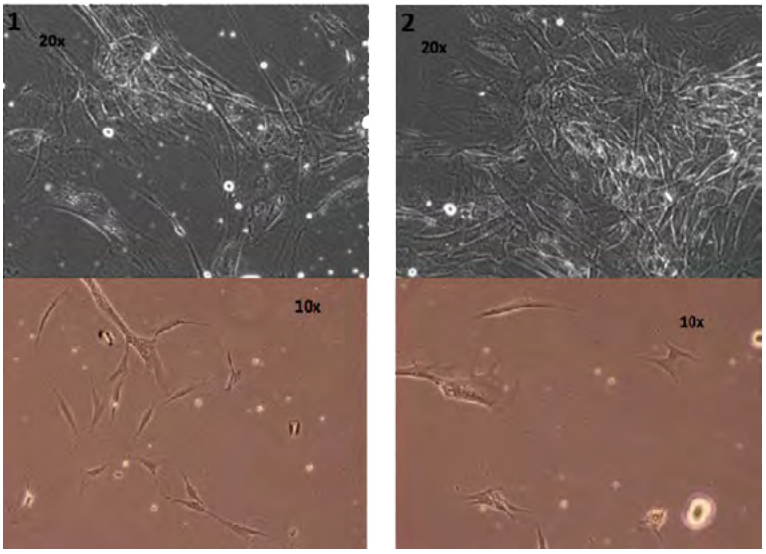


Figure 4. Photos of cellular suspension of the two patients by optical microscope.

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**).

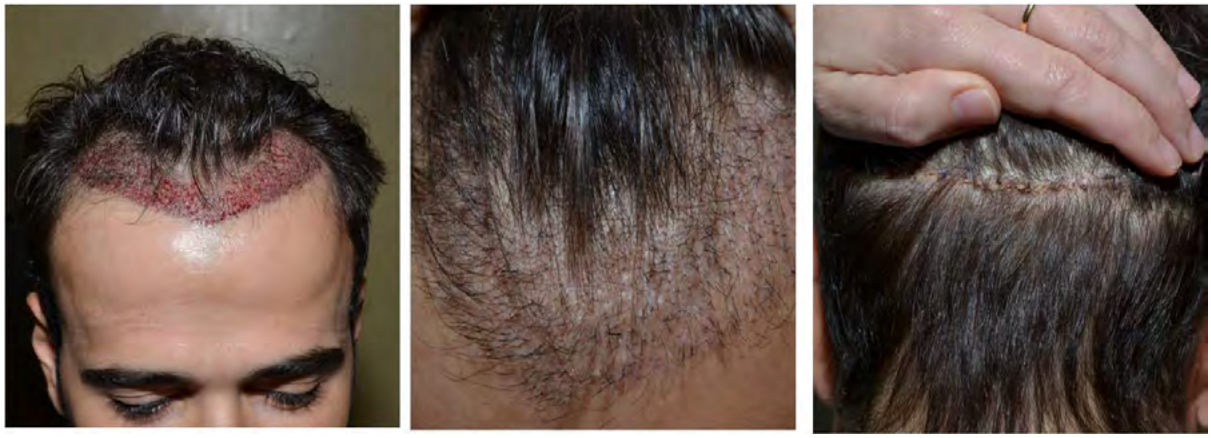
5.3. Patient's Impressions

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**).

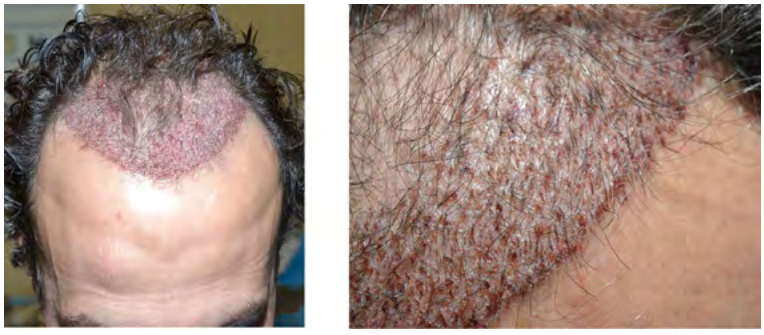
6. Discussion

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 can play its role in tissue regeneration.



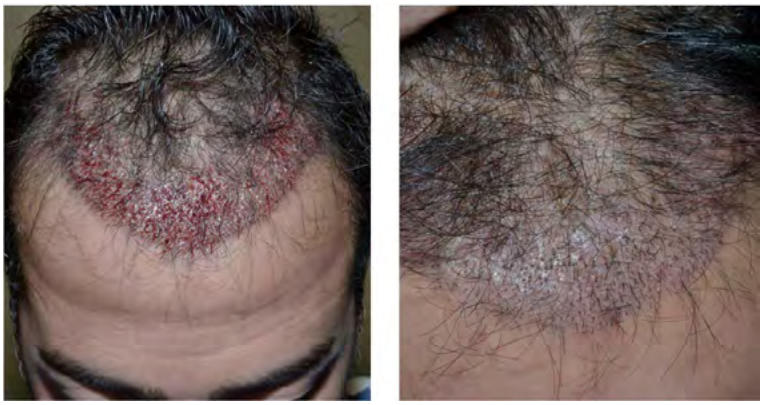
(a) (b) (c)

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.

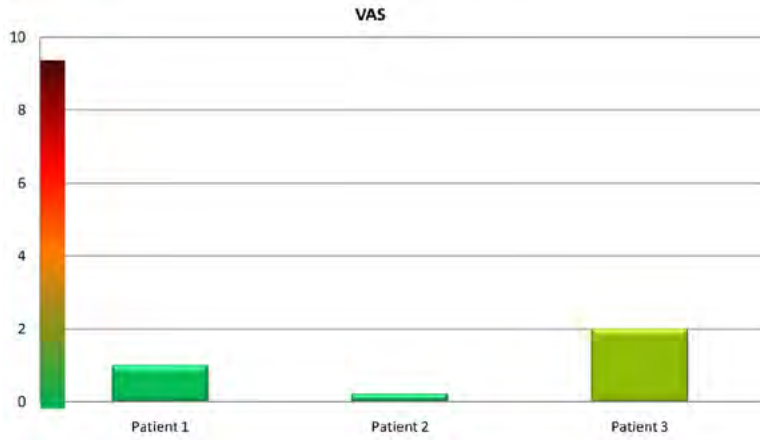


Figure 8. Visual analogic scale.

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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