Sequential Scalp Assessment in Hair Regeneration Therapy Using an Adipose-Derived Stem Cell–Conditioned Medium

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BACKGROUND An adipose-derived stem cell–conditioned medium (ADSC-CM) reportedly exerts skin-rejuvenating and hair growth-promoting effects. In the therapeutic application of ADSC-CM for alopecia, changes to the interfollicular scalp remain unclear although some evidence has indicated hair growth-promoting effects.

OBJECTIVE To evaluate the effects of ADSC-CM not only on hair follicles, but also on the interfollicular scalp.

METHODS Forty patients (21 men, 19 women; age range, 23–74 years) with alopecia were treated by intradermal injection of ADSC-CM every month for 6 months. Eighty fixed sites on patients were investigated by trichograms, physiological examinations, and ultrasonographic examinations at 4 time points (before treatment and 2, 4, and 6 months after the initial treatment).

RESULTS Hair density and anagen hair rate increased significantly. As physiological parameters, transepidermal water loss value gradually increased, with significant differences at 4 and 6 months after the initial treatment, but hydration state of the stratum corneum and skin surface lipid level showed no obvious changes. As ultrasonographic parameters, dermal thickness and dermal echogenicity were increased significantly.

CONCLUSION Intradermal administration of ADSC-CM on the scalp has strong potential to provide regenerative effects for hair follicles and the interfollicular scalp. An adipose-derived stem cell–conditioned medium offers a promising prospect as an alternative treatment for alopecia.

The authors have indicated no significant interest with commercial supporters.

Adipose-derived stem cells (ADSCs) are pluripotent mesenchymal stem cells within the stromal vascular fraction of subcutaneous adipose tissues. Because of the abundance, accessibility, and ease of isolation of these cells, ADSCs have gained popularity in the field of regenerative medicine.[1,2] Various cytokines secreted by ADSCs exert beneficial paracrine effects on surrounding cells and tissues.[3,4] An adipose-derived stem cells and conditioned medium of ADSCs (ADSC-CM) stimulates collagen synthesis and migration of human dermal fibroblasts (HDFs) during the wound-healing process.[5] An adipose-derived stem cell–conditioned medium protects HDFs from oxidative stress induced by chemical and ultraviolet B irradiation by inhibiting apoptotic cell death.[6,7] An adipose-derived stem cell–conditioned medium inhibits melanogenesis in B16 melanoma cells[8] and activates proliferation and migration of human keratinocytes.[9] These reports indicate potential for the application of ADSCs in the antiaging industry.

Hair growth-promoting effects mediated by ADSC-CM have also attracted much attention recently.

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TABLE 1. Summary of Patient Characteristics

Sex	*Stage*	*Patient Number*	*Age Range*	*Finasteride Administration Rate (%)*
Male	II, IIa	3	32–37	2/3 (66.7)
	III, IIIa, IIIv	6	26–40	6/6 (100.0)
	IV	1	53	1/1 (100.0)
	V, Va	3	26–57	3/3 (100.0)
	VI	8	31–65	7/8 (87.5)
	Total	21	26–65	19/21 (90.5)
Female	I	14	25–48	2/14 (14.3)
	II	5	23–74	1/5 (20.0)
	Total	19	23–74	3/19 (15.8)

Stage of alopecia was evaluated according to the Hamilton–Norwood scale for men and according to the Ludwig scale for women.

Among the numerous secretory factors released from ADSCs, hepatocyte growth factor (HGF) retards hair follicle regression and suppresses follicle keratinocyte apoptosis, contributing to anagen maintenance via Met receptor signaling.[10,11] Insulin-like growth factor-1 upregulates hair follicle growth through extracellular regulated kinase and Akt signaling pathways to regulate the anagen-to-catagen transition.[12,13] Vascular endothelial growth factor (VEGF) controls hair growth and follicle size by angiogenesis in a VEGF transgenic mouse model.[14] Platelet-derived growth factor (PDGF) induces and maintains the anagen phase through the Sonic hedgehog, Lef-1, and Wnt pathways[15] and provides the stem cell niche to regulate the hair cycle.[16] An adipose-derived stem cell-conditioned medium stimulates the growth of cultured human dermal papilla cells (DPCs) and the elongation of hair shafts in isolated hair follicles.[17] Subcutaneous administration of ADSC-CM induces the anagen phase and increases hair regeneration in C3H/NeH mice.[18] Therapeutic application of ADSC-CM is demanded as an alternative treatment for alopecia, whereas conventional treatments using finasteride and minoxidil are ineffective in some patients and difficult to apply for female patients.

Some clinical studies have shown the effectiveness and safety of alopecia treatment with ADSC-CM.[19–22] In those reports, improvements in patient satisfaction, hair density, and hair thickness were evaluated. Although ADSC-CM mediates skin-regenerative effects such as wound-healing, antioxidant protection, and antiwrinkling,[5–9] changes in the scalp skin initiated by alopecia treatment with ADSC-CM have not yet been evaluated. This study conducted sequential scalp assessments during ADSC-CM therapy for alopecia, aiming at evaluation of the effects of ADSC-CM not only on the hair follicles but also on the interfollicular scalp.

Methods

Patients

Subjects comprised 40 patients (21 men, 19 women; age range, 23–74 years) with androgenic alopecia or female pattern hair loss of varying severity (Table 1).[23–25] All patients were treated and evaluated at Cherry-Blossom Plastic and Regenerative Surgery, Tokyo, Japan. Histological examinations were performed at Tohoku University Graduate School of Dentistry, Sendai, Japan. Written informed consent was obtained from all patients before participation in this study, which was performed in accordance with the Declaration of Helsinki.

Treatments

AAPE (PROSTEMICS, Seoul, Korea) is a commercialized ADSC-CM product cultured under hypoxic conditions to enhance cytokine secretion from ADSCs.[9,18,26] The product is manufactured using the method described below.[9] Human subcutaneous adipose tissues were obtained from medical liposuction of healthy women after obtaining informed consent. Adipose tissues were exposed to 0.075% Type II collagenase (Sigma-Aldrich Corp, St. Louis, MO) for 30 minutes at culture temperature, centrifuged at 400*g* for 10 minutes, and then washed and resuspended in

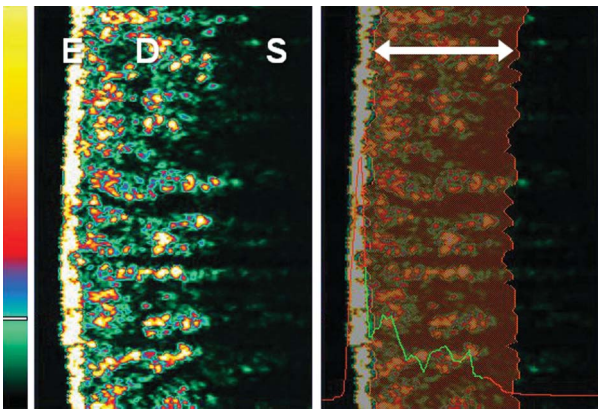

Figure 1. Representative ultrasonographic images from a 44-year-old woman. The intensity of reflected echoes is visualized as a color scale: white > yellow > red > blue > green > black. E = epidermal entry echo; D = dermis; S = subcutis. Double-headed arrow represents the area designated as the dermis. In these images, dermal thickness is 1.74 mm and dermal echogenicity is 33.1%.

phosphate-buffered saline. The stromal cell fraction was filtered through a 70-μm cell strainer (BD Biosciences, San Jose, CA). Using Histopaque-1077 (Sigma-Aldrich), ADSCs were isolated from the filtrate and then cultured at 37°C under 5% CO_2 in the Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Characteristic expression of stem cell–related surface markers and multilineage differentiation capability were confirmed using conventional methods.[5,6,27,28] After ADSCs were cultured and expanded in a normal control medium and frozen in aliquots using CellFreezer (Genenmed, Seoul, Korea) at passage 4, a frozen vial containing 1×10^6 cells was launched onto the culture medium containing 10% FBS and cultured to reach 5×10^8 cells. Introduced into CellFactory CF10 (Nunc, Rochester, NY) in a DMEM/F12 serum-free medium (Welgene, Taegu, Korea), cells were cultured under hypoxia by providing 2% O_2 using N_2 gas supply in a humidified multichannel incubator for 2 weeks. Conditioned media were collected and microfiltered, followed by quantitation of total protein production. Finally, 4-mL vials containing equal protein concentrations were freeze-dried for single-lot sample preparation of AAPE. AAPE contains various cytokines, including HGF, PDGF, and VEGF.[9] Although the product is not approved by the US FDA, the present authors have applied it in the treatment of alopecia with caution for 10 years.[19,20,22]

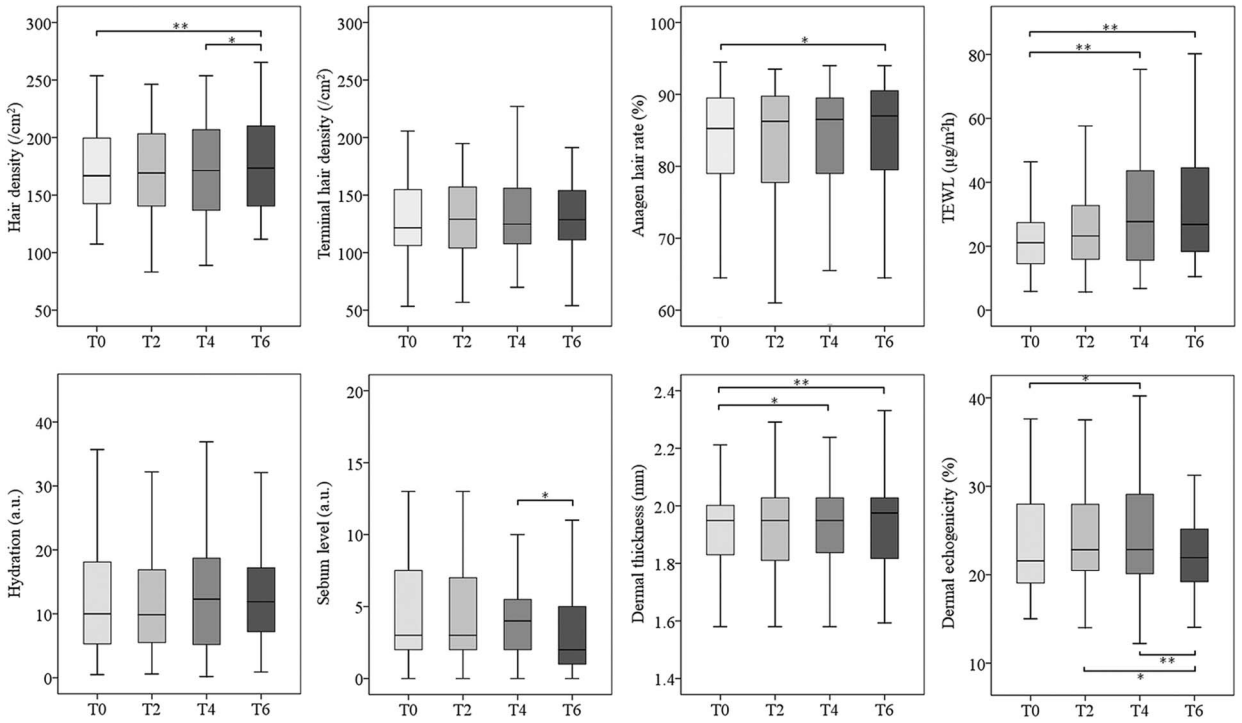

Figure 2. Time course of various parameters during ADSC-CM treatments. Data are shown in box-and-whisker plots. T0, beginning of study; T2, 2 months (status after 2 sessions of treatment); T4, 4 months (status after 4 sessions of treatment); T6, 6 months (status after 6 sessions of treatment). *$p < .05$; **$p < .01$. ADSC-CM, adipose-derived stem cell–conditioned medium.

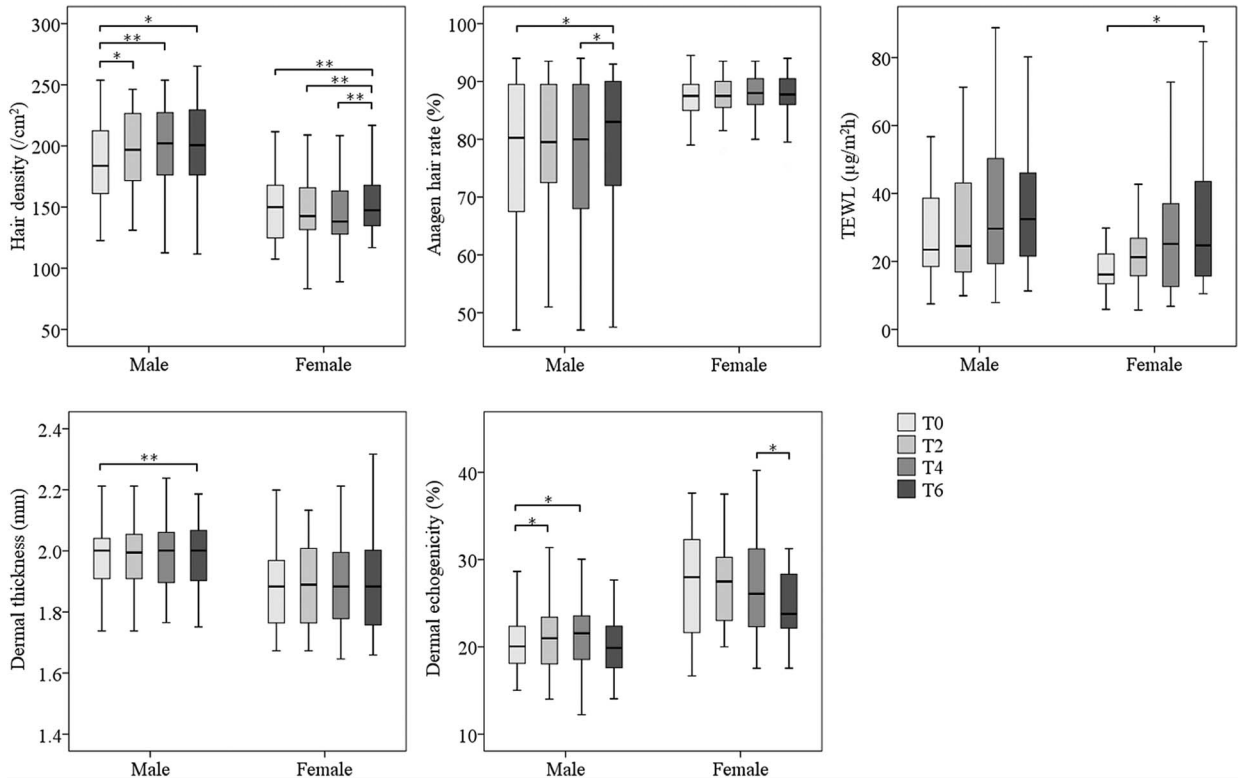

Figure 3. Time course of various parameters during ADSC-CM treatments evaluated in subpopulations according to sex. Data are shown in box-and-whisker plots. T0, beginning of study; T2, 2 months (status after 2 sessions of treatment); T4, 4 months (status after 4 sessions of treatment); T6, 6 months (status after 6 sessions of treatment). *$p < .05$; **$p < .01$. ADSC-CM, adipose-derived stem cell–conditioned medium.

Intradermal injections of AAPE were performed every month for 6 sessions. Some patients received additional sessions of treatments for further improvement after the study period. In each session, 1 vial of AAPE was dissolved in 4 mL of saline solution and injected to the entire scalp with a 31-G needle, as previously described.[19,20] No other topical or systemic therapies were performed without finasteride administration to 19 male patients and 3 female patients (Table 1).

Examination Protocol

Eighty sites in the 40 patients were examined at T0, just before the beginning of study treatment; T2, 2 months after the first treatment (status after 2 sessions of treatment); T4, 4 months after the first treatment (status after 4 sessions of treatment); and T6, 6 months after the first treatment (status after 6 sessions of treatment). Examination sites were determined as the intersection of a line extending cranially from the lateral angle of each eye and a line connecting both ears coronally, and each site was marked with a tattoo. In each examination, a 2-cm^2 area centered on this mark was clipped evenly 3 days before examinations.

Physiological Examinations

Before examinations, the hair was washed and dried in a constant manner. Patients were acclimatized for 15 minutes in another examination room adjusted to a temperature of 25°C and a relative humidity of 50%. Transepidermal water loss (TEWL) and hydration state of the stratum corneum were measured on the tattoo using ASA-MX100 (Asch Japan Co, Tokyo, Japan). The skin surface lipid level was measured using SM815 (Courage + Khazaka Electronic GmbH, Cologne, Germany).

Trichograms

Trichoscopic images were recorded using a digital camera (PowerShot 450; Cannon, Tokyo, Japan). The number of total hairs within a circle of 11 mm in diameter (area, 95 mm^2) centered on the tattoo was

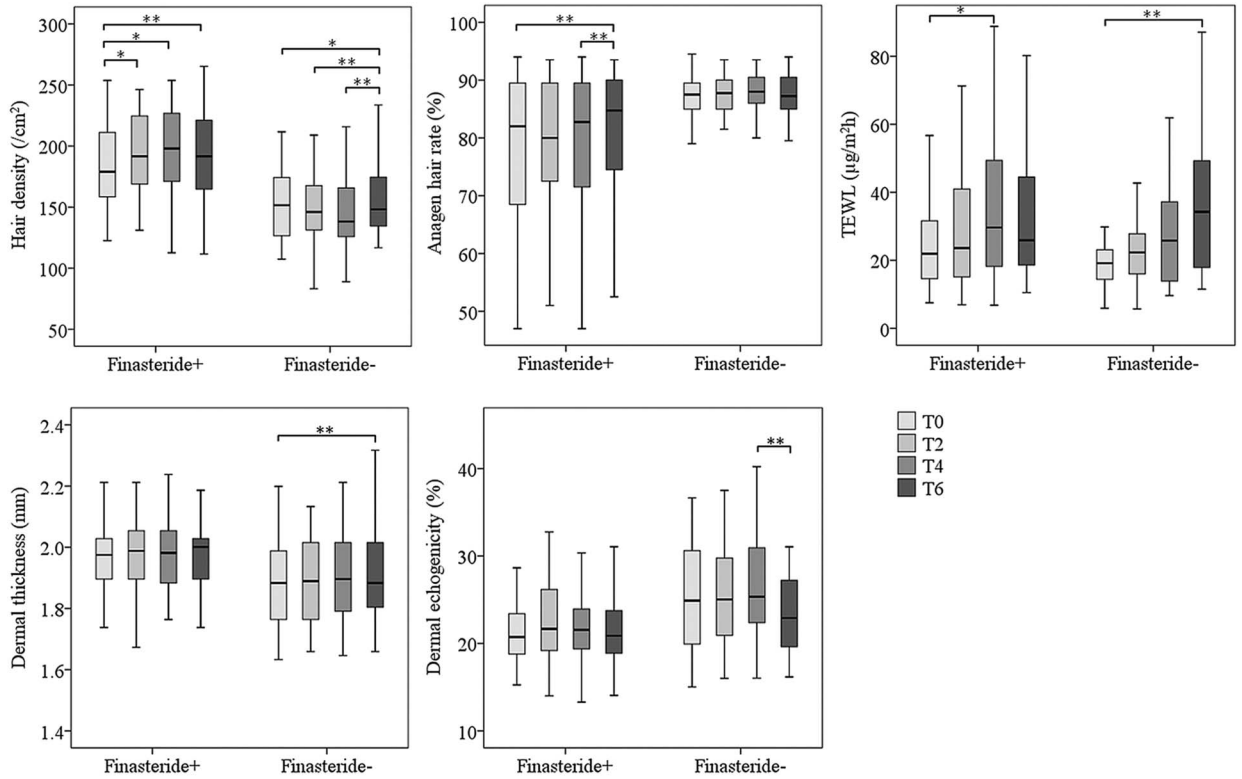

Figure 4. Time course of various parameters during ADSC-CM treatments evaluated in subpopulations according to presence or absence of finasteride administration. Data are shown in box-and-whisker plots. T0, beginning of study; T2, 2 months (status after 2 sessions of treatment); T4, 4 months (status after 4 sessions of treatment); T6, 6 months (status after 6 sessions of treatment). *$p < .05$; **$p < .01$. ADSC-CM, adipose-derived stem cell–conditioned medium.

counted by 2 technicians blinded to patients, and the mean of the 2 values was used for calculation of hair density. The terminal hair density and anagen hair rate were analyzed using TrichoScan Professional version 2.0 (DatInf GmbH, Tübingen, Germany).

Ultrasonographic Examinations

A cross-sectional image of the scalp at the tattoo was obtained using a 20-MHz ultrasound scanner (DremaLab; Cortex Technology, Hadsund, Denmark) by the same trained technician. The gain curve was standardized at 4 dB after preliminary studies. Dermal thickness was determined as the distance between the lower edge of the epidermal entrance echo and the interface between the dermis and subcutis. Dermal echogenicity, as the average amplitude of echoes in the area defined as the dermis, was calculated automatically (Figure 1). These measurements were performed by an evaluator blind to the patients. Two images were used for measurements at each site, and the mean of 2 values was used for subsequent analyses.

Histological Examinations

Punch biopsies (diameter, 3 mm) were taken from the scalp adjacent to the clipped area. Specimens were fixed with a 10% formalin solution, embedded in paraffin and sectioned at 4 μm. After the sections were subjected to staining with hematoxylin and eosin, and other sections were subjected to Elastica Masson–Goldner staining to facilitate the observation of collagen fibers (light green) and elastic fibers (dark purple). Sirius red/fast green staining (Picrosirius Red Stain Kit; Polysciences, Inc, Warrington, PA) was also used to identify Type I collagen (bright yellow orange) and Type III collagen (green) under a polarizing microscope.

Statistical Analysis

All statistical analyses were performed using SPSS software, version 23 (IBM, Armonk, NY). The normality of the authors' data distribution was assessed using the Shapiro–Wilk test. Because data were not normally distributed, nonparametric tests were

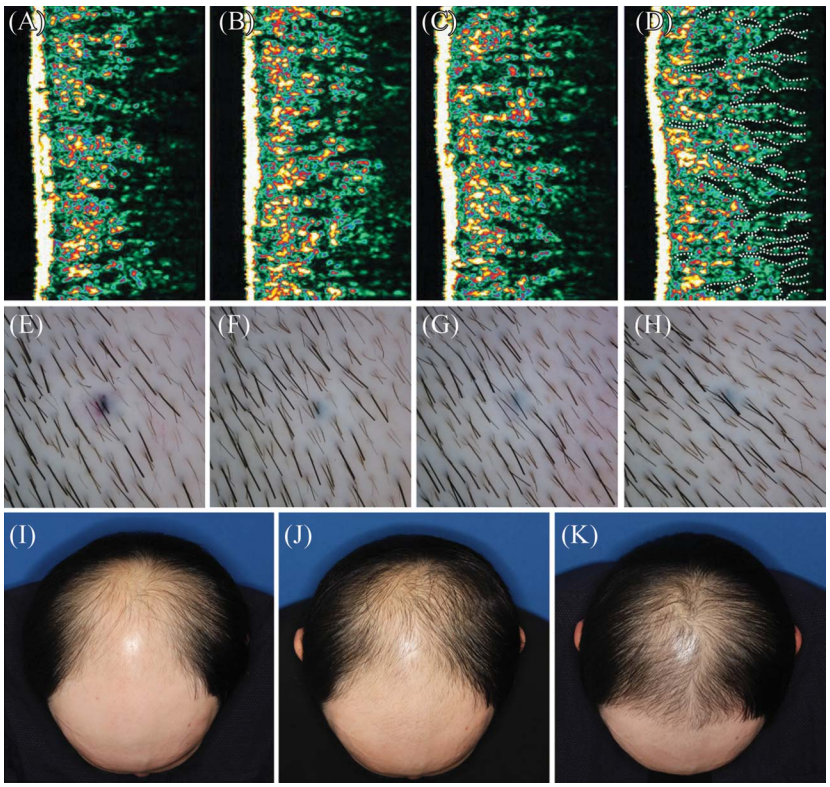

Figure 5. A 33-year-old man treated with ADSC-CM injection and oral finasteride. Upper row shows cross-sectional ultrasonographic images of the right scalp at T0 (A), T2 (B), T4 (C), and T6 (D). Dermal thickness increased from 2.05 mm (A) to 2.19 mm (D). Dermal echogenicity increased from 28.6% (A) to 32.2% (B), then decreased to 31.1% (D). Dotted line represents hair follicles (D). Middle row shows trichoscopic images of the right scalp at T0 (E), T2 (F), T4 (G), and T6 (H). Hair density increased from 159.5/cm^2 (E) to 176.3/cm^2 (H). Lower row shows clinical photographs at T0 (I), T6 (J), and 1 year after 10 sessions of treatment (K). ADSC-CM, adipose-derived stem cell–conditioned medium.

applied. The Friedman test and the Wilcoxon signed-rank test with Bonferroni correction were applied to compare results between the 4 groups of different time points. Statistical significance was defined as a value of $p < .05$.

Results

Overall results at 4 time points are presented in Figure 2. Among the hair growth parameters, the hair density and anagen hair rate improved significantly, whereas terminal hair density showed no significant differences during this study. Hair density increased from T0 to T6 ($p < .001$). The anagen hair rate increased from T0 to T6 ($p = .022$). Among the physiological parameters, TEWL gradually increased with statistical significance at T4 ($p = .007$) and T6 ($p = .009$), whereas the degree of stratum corneum hydration did not vary among the different time points. The skin surface lipid level showed a significant decrease from T4 to T6 ($p = .03$), but no difference from the baseline. Among the dermal parameters, dermal thickness assessed using ultrasonography showed a gradual increase with statistical significance at T4 ($p = .049$) and T6 ($p = .003$). Dermal echogenicity increased from T0 to T4 ($p = .022$) and decreased from T4 to T6 ($p = .001$).

Subgroup analysis was performed on some parameters across the subpopulations with different sex and finasteride administration (Figures 3 and 4). Trends in time-dependent changes in all populations, comprising (1) gradual increases in hair density, anagen hair rate, TEWL, and dermal thickness and (2) primary increase and subsequent decrease in dermal echogenicity, were similar to the trend in each subpopulation. Hair density increased significantly in all groups, but significant increases in the anagen hair rate and dermal thickness were limited to the male or finasteride groups. Transepidermal water loss increased significantly in the female group,

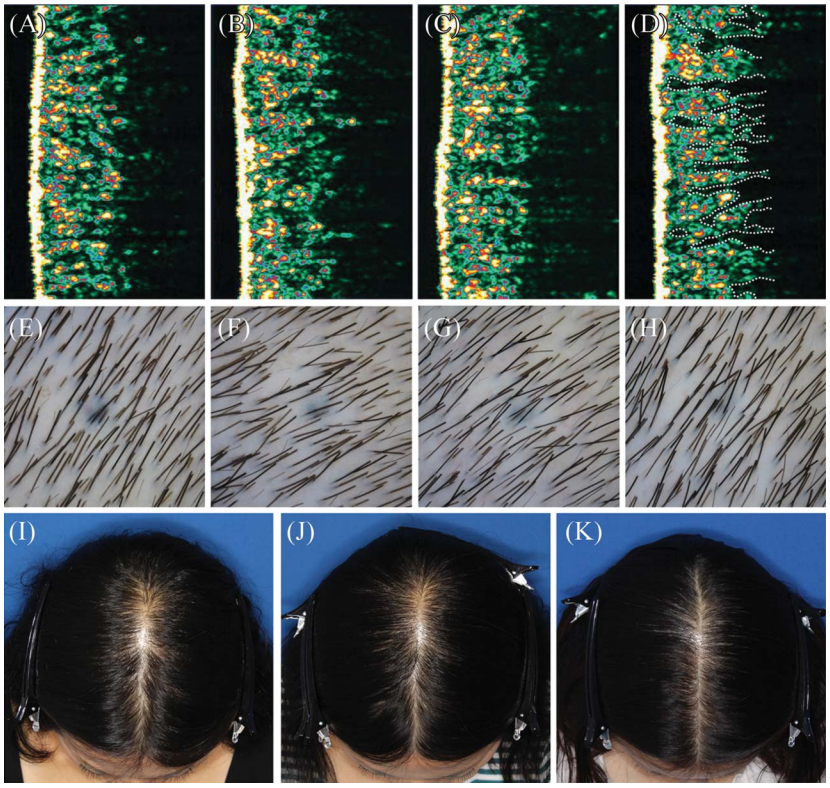

Figure 6. A 37-year-old woman treated with ADSC-CM injection. Upper row shows cross-sectional ultrasonographic images of the right scalp at T0 (A), T2 (B), T4 (C), and T6 (D). Dermal thickness increased from 1.69 mm (A) to 1.75 mm (D). Dermal echogenicity increased from 28.3% (A) to 31.8% (C), then decreased to 25.5% (D). Dotted line represents hair follicles (D). Middle row shows trichoscopic images of the right scalp at T0 (E), T2 (F), T4 (G), and T6 (H). Hair density increased from 143.2/cm^2 (E) to 149.5/cm^2 (H). Lower row shows clinical photographs at T0 (I), T6 (J), and 1 year after 8 sessions of treatment (K). ADSC-CM, adipose-derived stem cell–conditioned medium.

finasteride group, and nonfinasteride group. As for dermal echogenicity, a primary increase was significant in the male group and a subsequent decrease was significant in the female and nonfinasteride groups. Representative time courses for a male patient with finasteride administration and a female patient without finasteride administration are shown in Figures 5 and 6, respectively.

In 1 patient who was a 40-year-old man with androgenic alopecia Type II (Hamilton-Norwood classification) treated without finasteride, histological examinations were performed at T0, T4, and T6, which compared with ultrasonographic examinations. In histological examinations by Elastica Masson–Goldner staining, large collagen fibers (light blue) in the dermis seemed to increase at T4 and T6. Dark purple fibers, which denote elastic fibers, were not observed at all time points. In further examinations by Sirius red/fast green staining under polarizing microscopy, bright orange coloration of thick collagen fibers, which denotes Type I collagen, was predominantly observed at T4 and T6 (Figure 7).

Discussion

The present clinical study evaluated sequential changes to the hair and surrounding scalp skin elicited by serial ADSC-CM treatments. As a result, significant differences were observed not only in the hair growth parameters but also in cutaneous physiological parameters and dermal ultrasonographic parameters. Although inclusion of both patients with and without finasteride cotherapy is a limitation of this study, the authors performed subgroup analysis, and the results were similar to the trend in each subpopulation. To the best of the authors' knowledge, this represents the first report of scalp skin changes induced by ADSC-CM treatment for alopecia.

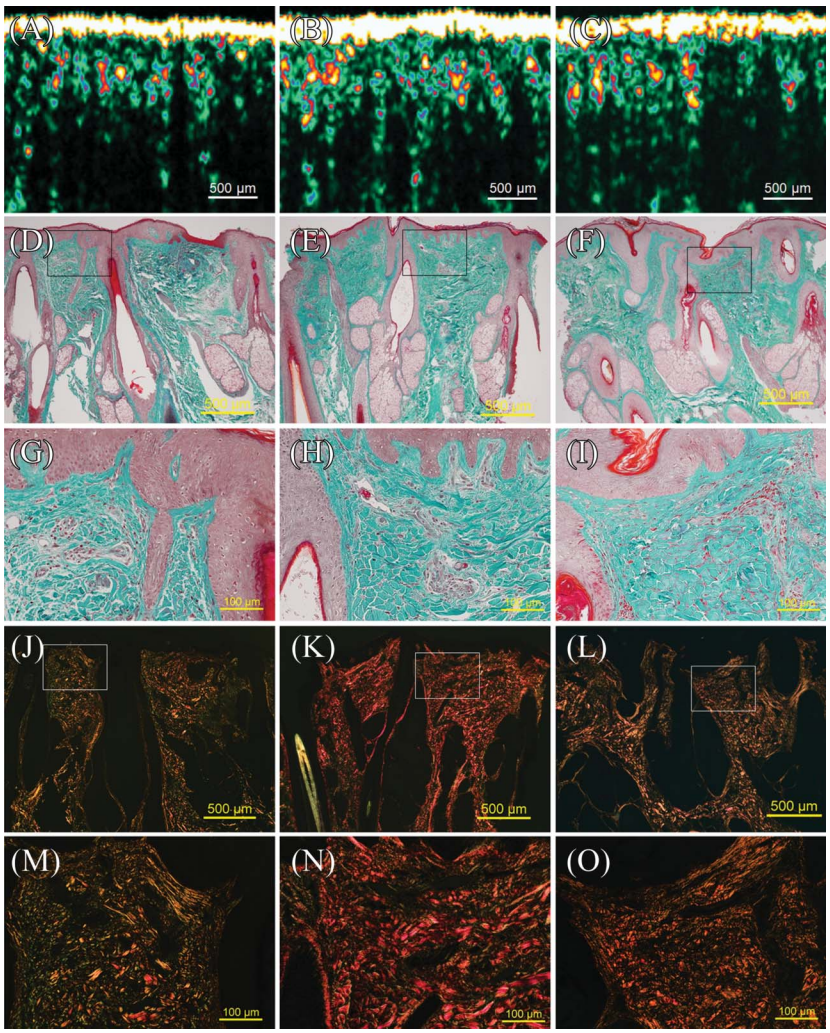

Figure 7. A 40-year-old man treated with ADSC-CM injection. Ultrasonographic images are processed by rotation and cropping (A–C), to facilitate comparisons with histologic images (D–O). Dermal echogenicity was 15.6% at T0 (A), 18.9% at T4 (B), and 16.8% at T6 (C). Elastica Masson–Goldner staining shows an increase in large collagen fibers (light blue) in the dermis from T0 (D and G) to T4 (E and H) and T6 (F and I). Sirius red/fast green staining under polarizing microscopy shows an increase in the bright orange color of thick collagen fibers, which denotes Type I collagen, from T0 (J and M) to T4 (K and N) and T6 (L and O). ADSC-CM, adipose-derived stem cell–conditioned medium.

Improvements in hair growth parameters confirmed the presence of hair growth-promoting effects of topical ADSC-CM administration, consistent with previous clinical reports.[20,21] Although terminal hair density did not show any significant changes, the authors got the impression that terminal hair growth gradually became evident after the study period and clinical improvement became clearer several months after the last ADSC-CM treatment, regardless of sex or finasteride administration (Figures 5K and 6K). The long-term efficacy of this treatment should be elucidated in a future study.

Skin is generally considered to dry with age, but no clear consensus has been reached regarding functional changes to scalp skin with age.[29,30] Florence and colleagues[30] reported decreased TEWL in the scalp of aged Caucasian women. A lower TEWL is commonly associated with more efficient skin barrier function. They discussed the possibility of a slowing scalp metabolism and lower scalp surface temperature, potentially associated with decreased vascularity in aging individuals. Increased TEWL in the scalp may suggest antiaging effects of ADSC-CM treatments, although the mechanisms remain unclear.

The skin in elderly individuals is typically thin and fragile, reflecting the fragmentation and reduction of Type I collagen fibrils, which comprise the bulk of the dermal extracellular matrix.[31,32] Ultrasonographic examination is a useful method for noninvasive, quantitative assessment of structural alterations in the skin with age[33–35] and antiaging therapies.[36,37] Significant increases in dermal thickness and echogenicity are compatible with findings from animal studies, where increased dermal thickness and collagen contents in the dermis after ADSC-CM administration have been observed in histologic and Western blot studies.[7,38] However, reasons for the decreases in dermal echogenicity seen from T4 to T6 were unclear. Given one case of comparing ultrasonographic and histological changes, the increase in echogenicity from T0 to T4 seems to agree with increased presence of collagen fibers in the dermis, but the decreased echogenicity from T4 to T6 seems to contradict the incremental maintenance of collagen fibers (Figure 7). On the other hand, vertical hypoechoic lines in the dermis, which implied hair follicles, were marked at T6 (Figures 5D and 6D). Taken together, the growth of hair follicles might be linked to the high inclusion rate of hair follicles in the cross-sectional image and reductions in dermal echogenicity, counter to the relatively unchanged amount of collagen fibers in the dermis.

Despite the limited histological confirmation and absence of negative controls, the authors' findings revealed significant changes in TEWL, dermal thickness, and dermal echogenicity in the scalp treated by ADSC-CM, possibly suggesting regenerative changes in the scalp skin. In addition, these changes became evident 2 months earlier than the significant increases in the hair density and anagen hair rate. Festa and colleagues[16] reported that adipocyte precursor cells in murine skin proliferate during late catagen (i.e., before anagen) and immature adipocyte lineage cells are necessary and sufficient to drive follicular stem cell activation. Such evidence could support the authors' speculation that ADSC-CM administration promotes hair growth through the gradual spread of paracrine effects on the interfollicular dermis, as well as paracrine effects directly on follicular DPCs.

Conclusion

The authors' clinical assessment suggests that ADSC-CM may prove effective for the regeneration of hair follicles and interfollicular scalp. The latter effect may also enhance hair growth. An adipose-derived stem cell–conditioned medium offers promise as an alternative treatment for alopecia.

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