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Randomized trial of electrodynamic microneedling combined with 5% minoxidil topical solution for treating androgenetic alopecia in Chinese males and molecular mechanistic study of the involvement of the Wnt/β-catenin signaling pathway

Running title: Treating AGA with Microneedling and Minoxidil

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Abstract

Background: Treatment of androgenetic alopecia (AGA) with concurrent electrodynamic microneedling and 5% minoxidil may further stimulate hair growth.

Objectives: To evaluate the efficacy of microneedling combined with 5% minoxidil in Chinese male AGA patients and to explore the underlying mechanisms.

Methods: Seventy-one male volunteers with AGA completed the entire trial and follow-up. The first group (n=23) received only 5% minoxidil twice daily for 24 weeks; the second group (n=23) received only microneedle therapy every 3 weeks for 8 treatments; and the third group (n=25) received the combination treatment for a total of 24 weeks. Changes in hair density and diameter were evaluated before and after treatment every 3 weeks, and patients were followed up at 6 months after the final treatment. In the combination group, a PCR array was used to detect the expression of molecules in the Wnt/β-catenin pathway within the hair loss sites on top of the head before and after treatment and within the scalp tissues from non-hair loss sites on top of the head. The tissues were obtained by punches in the most severe area of hair loss on top of the head and in the adjacent normal hair area without hair loss. Real-time quantitative PCR and western blotting were used to further examine changes in the differentially expressed molecules identified by PCR array (FZD3) and in molecules in the Wnt/β-catenin signaling pathway closely related to hair growth (β-catenin and LEF-1).

Results: Compared to single minoxidil or single microneedle treatment, the combination therapy showed superior therapeutic effects clinically, with further upregulation of FZD3, β-catenin, and LEF-1 expression levels at both mRNA and protein levels in the treated areas.

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Conclusions: Microneedling combined with 5% minoxidil can improve AGA, and the underlying mechanism may involve activation of the Wnt/β-catenin signaling pathway.

Keywords: Androgenetic alopecia, microneedle, drug delivery, Wnt/β-catenin signaling pathway

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Introduction

Androgenetic alopecia (AGA) mainly occurs in men aged 20–55 years old, with the following main manifestations: decreased hair density both sides of the forehead[1]; thinning of hair gradually extending to the top of the head[1-3]; receding of the front hairline[4]; and an M-shaped front hairline[5, 6]. Although AGA does not affect the health of the body, it has received attention due to its detriment to the patient's self-image[7]. A variety of methods can be applied to treat AGA, including oral finasteride, topical minoxidil, phototherapy, and hair transplantation[5, 8-10]. However, no single method has been determined to be the most effective. At present, many researchers are trying to improve AGA treatment. Microneedle treatment combined with topical minoxidil is considered promising for the treatment of hair loss, but there have been few relevant studies[11].

The signal transduction pathway that is most closely related to the pathogenesis of AGA is the Wnt/β-catenin pathway[12]. Activation of this signaling pathway is essential for hair follicle development, hair growth, and the hair cycle[12]. The maintenance of hair follicle function requires the interaction of the Wnt signaling pathway between the epidermis and dermis[5, 12]. It increases the size and number of hair follicles and maintains the environment during the anagen phase of the hair follicle[13].

Currently, most AGA studies have evaluated the clinical efficacy of treatment based on qualitative descriptions, while the mechanistic studies have focused on animal experiments. Thus, in-depth studies on the quantitative effects of treatments and underlying molecular mechanisms within human tissues are lacking. Therefore, we designed this study and observed the effect of

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microneedle treatment combined with 5% topical minoxidil for treating male AGA patients and its efficacy and safety through objective evaluation. We used polymerase chain reaction (PCR) arrays to identify differentially expressed messenger RNAs (mRNAs) in the Wnt/β-catenin pathway to elucidate a possible pathogenic mechanism of AGA. Subsequently, after determining the mRNAs and proteins in the Wnt/β-catenin signal transduction pathway that were significantly differentially expressed and closely related to the hair growth cycle, their expression levels in scalp tissues from the different groups were compared before and after treatment to provide a theoretical basis for the clinical efficacy of the treatment.

Materials and methods

The protocol for the overall trial was reviewed and approved by the Institutional Review Board at The First Affiliated Hospital of China Medical University. Also, the research was performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent before their inclusion in the study.

Patients and treatment regimen

At the Department of Dermatology of The First Affiliated Hospital of China Medical University, Shenyang, China, 20-to-60-year-old male participants diagnosed with Norwood-Hamilton type III-VI AGA were enrolled in this study. In line with the instructions for the study, the participants cut their hair on a regular basis so that the length and style of their hair was unchanged throughout all of the follow-up visits. The present study encouraged participants

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to not dye their hair unless it had been dyed prior to recruitment. This study excluded men who had received any medications during the preceding 24 months or had demonstrated abnormalities in their laboratory test results or on physical examination. Additionally, except for the drugs used in the research, the included patients did not receive any treatments known to stimulate hair growth. The participants were randomly divided into three groups according to the random number table approach: the topical 5% minoxidil group (group 1, n=23), the electrodynamic microneedle treatment group (group 2, n=23), and the combination of topical 5% minoxidil and electrodynamic microneedle treatment group (group 3, n=25). Subjects in groups 2 and 3 received microneedle treatments every 3 weeks for a total of 8 treatments. Using PASS 15.0 software, we calculated the sample size required for the clinical observation in this study. The results showed that at least 66 samples were required for statistical analyses of the three groups, and our study included 71 cases (originally 75, with 4 dropouts).

Procedures

Participants were instructed to wash their hair prior to the microneedle treatments and to have their hair cut every 6 weeks. Alcohol wipes were used to clean the treatment sites three times prior to the therapies. Anaesthetic ointment was also used before the treatments. During treatments, an electrodynamic microneedle (Yuanxiang Biological Technology Company, Guangzhou, China) was inserted at a depth of 1–2 mm. The treatment endpoints were hemorrhage and redness at the treatment site. In group 3, 2 ml of 5% minoxidil (Wansheng Pharmaceutical Company, Hangzhou, China) was topically used simultaneously with the

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microneedle treatments. Subsequently, to enhance the absorption of the drug, the scalp was massaged. In contrast, participants in group 2 solely underwent microneedle treatments without minoxidil. They were told to not wash their hair for 24 h after the treatment. Patients in groups 1 and 3 topically applied 1 ml of 5% minoxidil solution twice daily throughout the study, except on the microneedle treatment days in group 3 because the same amount of minoxidil had already been used during the treatments.

Outcome measures

Participants underwent evaluation every 3 weeks, and at the same time, the details for any toxicities related to treatments for all groups were collected. Additionally, participants were asked to return for a follow-up visit 6 months after the completion of treatments for evaluation of their hair growth after the treatments.

Bald areas at baseline, after 24 weeks of treatment, and 6 months after the end of treatment were the primary outcome of the clinical trial. A high-pixel digital camera (D610, Nikon, Japan) was used to photograph the bald spots during every follow-up visit. During every photo session, the positioning and posture of the patients, position and angle of the camera, and lighting conditions were recorded. From the baseline until week 24, microscopic photographs were taken for analysis every 3 weeks. When the contact dome was applied to the head of each patient, the same 1-cm^2 zone of the bald area was photographed. We used the anatomical landmark method to record the detected bald area (the detected area was the intersection point of fixed distances from multiple anatomical landmarks; according to the Hamilton-Norwood

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classification, different types of AGA have different anatomical landmarks, and the position of the landmark of each patient was recorded in detail). Additionally, before photographs were taken, hair in that zone was cut to a length of 1–2 mm. Areas of AGA that were followed for response were marked by keeping the hair 1–2 mm in length over the course of the study to further ensure that the same bald spot was detected during the research, as tattoos are not prevalent in China.

This study employed a hair microscope system (CBS-1717, Boshi Electronics, Taiwan, China) to detect the thickness and density of hair, which were the secondary outcomes of the trial. The system contains a camera and a computer. The detector measures the counts and the diameters of hairs per square centimeter on the scalp at 70× magnification in every microscopic photograph. The density of hair was recorded as the number of hairs per cm^2. Hair less than 40 μm in diameter was regarded as vellus hair, and hair greater than 40 μm in diameter was regarded as non-vellus hair, as described previously[14, 15]. The total counts of hairs were the sum of vellus hairs and terminal hairs. The numbers and diameters of hair were counted by three board-certified dermatologists with no previous knowledge of the treatments. Non-vellus hair was employed to record the hair thickness.

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RT^2 profiler PCR array tests

For the male AGA patients receiving microneedle treatment combined with topical minoxidil, the molecular expression levels of the Wnt/β-catenin signal transduction pathway in scalp tissues from the hair loss sites on the top of the head before and after treatment and in the

scalp tissues from the non-hair loss sites on top of the head were compared. Real-time quantitative reverse-transcription PCR (qRT-PCR) was used to validate the results. These volunteers included 9 unrelated male AGA patients between 30 and 60 years old. The hair loss type was Norwood-Hamilton type V-VI AGA. The volunteers had not received any prior treatment, and their history of hair loss was more than 8 years. The sites for scalp tissue samples collected before the treatment were in the most severe area of hair loss on the top of the head (with follicle density $<150/cm^2$) and in the adjacent (>5 cm) normal hair area without hair loss (with follicle density $>325/cm^2$). The scalp tissues were obtained by punches of 5 mm in diameter and 5 mm in depth and then frozen in liquid nitrogen within 3 min after the tissues were sampled. The scalp tissues from the hair loss area underwent histological examination to confirm the diagnosis. The sites of scalp tissue samples collected after the treatment were in the treated area, and the tissues were taken 3 weeks after the last treatment. Three sets of scalp tissues from the hair loss sites before and after treatment and the non-hair loss sites were used for gene pathway analysis, while the other 6 pairs of scalp tissues from the hair loss and non-hair loss sites before treatment were used for subsequent validation.

This study employed a custom RT^2 Profiler PCR Array (PAHS-043Z, Qiagen, Germany). It serves to synchronically identify 84 genes associated with the Wnt signaling pathway in humans. Reverse transcription, positive PCR controls, and genomic DNA contamination were included in every 96-well set in every plate. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the assay reference gene. Using TRIzol reagent (Qiagen, Germany), total RNA was separated from every frozen tissue sample and quantified using a Nanodrop 2000

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(Thermo Fisher Scientific, USA). Subsequently, total RNA (with the amount of 500 ng) was reverse transcribed to an ultimate volume of 10 μL using the RT^2 First Strand Kit (Catalog No. 330401, Qiagen, Germany). The RT^2 Profiler PCR Array tests were carried out according to the manufacturer's instructions. The cDNAs in every sample (10 μL) were diluted in RNase-free water in a final volume of 111 μL. Subsequently, we mixed 102 μL of the diluted cDNAs with 1248 μL of RNase-free water and 1350 μL of 2× RT^2 SYBR Green qPCR Mastermix (Catalog No. 330529, Qiagen, Germany). Twenty-five microliters of the mixture above was included in every well of the 96-well array plate. qPCR analysis was completed using the Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems, USA) with the following thermal cycling parameters: 10 min at 95°C, and then 40 cycles of 15 sec at 95°C and later 1 min at 60°C. We input the exported Ct values to an Excel template file offered by SABiosciences (Qiagen, Germany). Subsequently, it was uploaded for analysis online, and qualified statistics data for 9 samples were analyzed using the $2^{-\Delta\Delta Ct}$ method. Fold changes in expression were calculated by dividing the normalized gene expression in the test sample (the hair loss site on top of the head after treatment and the non-hair loss site on top of the head) by that in the control sample (the hair loss site on top of the head before treatment). The data analysis was performed based on the $2^{-\Delta\Delta Ct}$ values. Only Ct values <35 were included in the calculations. The fold change values for gene expression were analyzed online using Qiagen's web-based PCR Array data analysis software. Statistically significantly up- or down-regulated genes were determined based on fold change values greater than 1.5 or less than -1.5, respectively.

Next, we selected the molecules with the highest differential expression in the

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Wnt/β-catenin signaling pathway screened by the PCR array for qRT-PCR analysis and further verified their differential expression levels in this pathway (6 pairs of scalp tissues from the hair loss and non-hair loss sites before treatment were compared). Primers used in qRT-PCR for quantification of the selected mRNA were designed and synthesized by Qiagen Corporation (Germany). The expression of the selected mRNAs was normalized to GAPDH. A 7900 Real-time PCR System (Applied Biosystems, USA) was used for qRT-PCR analysis. The PCR conditions were 95°C for 2 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Samples were assayed in triplicate. The formula for the relative fold change in gene expression was: $2^{-\Delta\Delta Ct}=2^{-[\Delta Ct(\text{hair loss site before treatment})-\Delta Ct(\text{non-hair loss site before treatment})]}$, where ΔCt=Ct(detected gene)-Ct(GAPDH), and Ct represents the threshold cycle number.

Detection of frizzled class receptor 3 (FZD3), β-catenin, and lymphoid enhancer binding factor 1 (LEF-1) mRNA expression by qRT-PCR

qRT-PCR was used to further detect the FZD3, β-catenin, and LEF-1 mRNA expression before and after treatment in each group of scalp tissues. FZD3 showed the highest differential expression in the PCR array results in the Wnt/β-catenin pathway, and β-catenin and LEF-1 were significantly related to AGA hair loss in the Wnt/β-catenin pathway. The scalp tissues from hair loss sites before and after treatment from nine volunteers in each treatment group were collected. The sites and time for scalp tissue collection were the same as those in the previous section.

Primers used in qRT-PCR for quantification of the selected mRNAs were designed and synthesized by Qiagen Corporation (Germany). The expression of the selected mRNAs was

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normalized to that of GAPDH. The method was the same as described above. For FZD3, the forward primer sequence was 5-AGCCGACAGGTACTCCAGGAAC-3 and the reverse primer sequence was 5-CGTGACATGCTGCCATGAGGTAG-3. For β-catenin, the forward primer sequence was 5-ATGGCCATGGAACCAGACAG-3 and the reverse primer sequence was 5-CAGGGAACATAGCAGCTCGT-3. For LEF-1, the forward primer sequence was 5-TTCTCCACCCATCCCGAGAA-3 and the reverse primer sequence was 5-CTGATGGGATGTGTGACGGG-3. For GAPDH, the forward primer sequence was 5-TCAAGGCTGAGAACGGGAAG-3 and the reverse primer sequence was 5-TGGACTCCACGACGTACTCA-3.

Detection of FZD3, β-catenin, LEF-1 protein expression by western blotting

Western blotting was used to further detect changes in the protein expression levels of FZD3, β-catenin, and LEF-1 from nine volunteers in each treatment group before and after treatment. The sites and time for scalp tissue collection were the same as described above.

Protein preparation

After separation from scalp tissues, total protein was suspended in chilled lysis buffer (1×Tris-buffered saline, 1.5% Triton X-100, 0.5% deoxycholic acid sodium salt, 0.1% sodium dodecyl sulfate [SDS], protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). We homogenized the tissues using lysis buffer, and the homogenates were kept on ice for 5 min and subsequently centrifuged (Centrifuge Z 32 HK, HERMLE Laboratory Technology

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GmbH, Wehingen, Germany) at 12,000 rpm and 4°C for 10 min. We collected the supernatants for use in western blotting. In every sample, the aggregate protein concentration was tested using a BCA protein assay reagent kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol.

Western blotting analysis

We solubilized total proteins in Laemmli sample buffer for 5 min at 70°C for use in DS-polyacrylamide gel electrophoresis. After transfer to polyvinylidene difluoride (PVDF) membranes (Beyotime Biotechnology, Shanghai, China), for western blotting membranes were stripped, isolated, and incubated overnight at 4°C with anti-LEF-1 antibody (Abcam, UK), anti-FZD3 antibody (Abcam, UK), anti-β-catenin antibody (Abcam, UK), or anti-GAPDH antibody (Abcam, UK).

After being washed, membranes were incubated with anti-mouse secondary antibody (ZSGB-BIO, Beijing, China) or horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody at 37°C for 45 min. In addition, the membranes were visualized by enhanced chemiluminescence (ECL, Beyotime Biotechnology, Shanghai, China).

Western blots were scanned using an imaging system (Bio-Rad Laboratories, USA), and the labeling density was quantified. Densitometry results are reported as volume-integrated values and expressed as fractions compared with the mean value of controls, corrected by GAPDH protein concentration.

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Statistical analysis

SPSS 22.0 software was used for the statistical analyses and processing. $P<0.05$ was considered statistically significant. One-way analysis of variance (ANOVA) was used to compare the observed differences between three trial groups. Student's *t* test was used to compare the differences between either set of two groups.

Western blot analysis was conducted using ImageJ software (National Institutes of Health, USA) to analyze the grey values of the protein bands. During the analysis, the ratio of the grey values for the proteins of the target genes and the internal reference genes of the same sample was calculated as the relative content of the protein. In the PCR array tests, PCR array software was used for data processing, and the Wilcoxon nonparametric rank-sum test was used.

Results

Study population

The 75 patients were randomly placed into three treatment arms (n=25 each; Fig. 1). The patients in the three treatment groups were characterized by similar age, duration of hair loss, and hair density at the start of the study (Table 1). Twenty-three patients in group 1, twenty-three patients in group 2, and all twenty-five patients in group 3 completed the study and had data available for efficacy analysis. Data for all 75 patients were available to assess toxicity.

[Insert Fig. 1 near here]

[Insert Table 1 near here]

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Clinical study efficacy

We compared the non-vellus hair density in each group at the observation endpoint (3 weeks after the last treatment) with the non-vellus hair density before treatment and found that all three groups showed significantly increased non-vellus hair density ($P<0.05$). The elevations of non-vellus hair density of group 2 (32.09±4.51 roots/cm^2) was higher than that of group 1 (28.01±7.42 roots/cm^2), and the highest elevation of non-vellus hair density was observed in group 3 (56.45±7.82 roots/cm^2) at the observation endpoint ($P<0.001$, one-way ANOVA). Group 2 did not show a markedly elevated final hair density in comparison to group 1; group 3 showed superior final hair density compared to either group 1 or group 2 at the observation endpoint ($P<0.001$, Table 2).

[Insert Table 2 near here]

The variations of vellus hair density of group 1, group 2 and group 3 at the observation endpoint were -13.74±3.41 roots/cm^2, -10.56±2.83 roots/cm^2 and -16.67±6.48 roots/cm^2, respectively. We then compared the vellus hair density of each group at the observation endpoint with the vellus hair density before treatment and found that group 1 and group 3, but not group 2, showed significantly decreased vellus hair density ($P<0.05$). Neither one-way ANOVA for the three groups nor paired comparisons between either two groups showed significant differences in the variation of vellus hair density at the observation endpoint (Table 2).

We finally compared the final hair diameter of each group at the observation endpoint with the final hair diameter before treatment and found that group 1 and group 3, but not group 2, showed a significantly increased final hair diameter ($P<0.001$). The increases in final hair diameter

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of group 1, group 2, and group 3 at the observation endpoint were 18.70±6.17 μm, 10.41±4.83 μm, and 22.46±3.87 μm, respectively, showing a statistically significant difference (P=0.002) as tested by one-way ANOVA. We further made paired comparisons between either two groups and found that the elevation of final hair diameter of group 1 and group 3 outperformed that of group 2 (P=0.015 and P<0.001), but there was not a significant difference between group 1 and group 3 (Table 3).

[Insert Table 3 near here]

Six months after the end of the treatment, the volunteers returned for follow-up (Fig. 2). Ninety percent of the volunteers treated with only minoxidil had recurrence of hair loss, and all newly formed hair was lost. For the group that received only microneedle treatment and the group that received the combination treatment, the newly formed hair was maintained very well: 10% of the volunteers lost all the new hair, 70% of the volunteers had a small amount of hair loss, and 20% of the volunteers maintained the increased hair density after treatment.

[Insert Fig. 2 near here]

Toxicity

During treatment, 12 volunteers had adverse reactions. In group 1, 3 volunteers had adverse reactions, including 1 case of seborrheic dermatitis, 1 case of scalp itching, and 1 case of eczema. In group 2, 4 volunteers had adverse reactions, including 2 cases of increased dandruff, 1 case of scalp infection, and 1 case of enlarged lymph nodes in the neck or behind the ear. In group 3, 5 volunteers experienced adverse reactions, including 1 case of seborrheic dermatitis, 1 case of

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increased dandruff, and 3 cases of enlarged lymph nodes in the neck or behind the ear. There was no significant difference in the occurrence of adverse reactions among the three groups.

PCR array analysis of gene expression related to the Wnt/β-catenin signaling pathway

For group 3, the expression levels of genes involved in the Wnt/β-catenin signal transduction pathways in the scalp tissues from the hair loss sites before and after treatment and the scalp tissues of the non-hair loss sites on the top of the head were compared using PCR array analysis. Compared with the hair loss sites, the non-hair loss sites showed upregulation of 16 differentially expressed genes (DEGs) and downregulation of 2 DEGs in the Wnt/β-catenin pathway; FZD3 was upregulated by 2.45-fold (P=0.001598). Compared with before treatment, after treatment scalp tissues at the sites of hair loss showed upregulation of 8 DEGs and downregulation of 3 DEGs; FZD3 was upregulated by 1.85-fold (P<0.0000001). Compared with hair loss sites before treatment, scalp tissues at non-hair loss sites showed upregulation of 4 of the same DEGs identified at sites of hair loss after treatment (but no downregulated DEGs), including FZD3 (Tables 4 and 5).

[Insert Table 4 near here]

[Insert Table 5 near here]

Considering both the high fold change and low P value for differential expression of FZD3 at both non-hair loss sites and hair loss sites after treatment in comparison to hair loss sites without treatment, we further validated the alteration of FZD3 expression in scalp tissues using qPCR. We found that the expression levels of FZD3 in the scalp tissues from non-hair loss sites (8.97±1.47)

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were significantly higher than those in the scalp tissues from hair loss sites (1.69±0.66, P<0.05, Table 6).

[Insert Table 6 near here]

mRNA expression levels of FZD3, β-catenin, and LEF-1 by qRT-PCR

Using qRT-PCR, we found that the FZD3 and β-catenin expression levels in the scalp tissue at the observation endpoint were significantly increased in group 1 and group 3 (P<0.05), but decreased (not significant) in group 2 compared to those in the scalp tissue before treatment. FZD3 and β-catenin were differentially expressed in all three groups (P<0.05, one-way ANOVA). FZD3 expression in group 3 was significantly higher than that in group 1 and group 2 (P<0.05); β-catenin expression in group 1 was significantly higher than that in group 3 (P < 0.05); and β-catenin expression in group 3 was significantly higher than that in group 2 at the observation endpoint (P<0.05, Fig. 3, Table 7).

[Insert Fig. 3 near here]

[Insert Table 7 near here]

LEF-1 expression levels in the scalp tissue at the observation endpoint increased in all three groups, but only group 3 showed significant elevation compared to that in the scalp tissue before treatment (P<0.05). LEF-1 was differentially expressed by all three groups (P<0.05, one-way ANOVA) with LEF-1 expression in group 3 being significantly higher than that in group 1 or group 2 (P < 0.05), and LEF-1 expression in group 2 was significantly higher than that in group 1 at the observation endpoint (P<0.05, Fig. 3, Table 7).

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Western blot analysis of the expression levels of FZD3, β-catenin, and LEF-1

Using western blotting, we found that FZD3 expression in the scalp tissue at the observation endpoint was significantly increased in group 2 and group 3 ($P<0.05$), but decreased (not significant) in group 1 compared with that in the scalp tissue before treatment. FZD3 was differentially expressed by all three groups at the observation endpoint ($P<0.05$, one-way ANOVA).

[Insert Fig. 4 near here]

[Insert Fig. 5 near here]

[Insert Table 8 near here]

β-catenin expression in the scalp tissue at the observation endpoint was increased in group 2 and group 3, but only group 3 showed a significant elevation compared with the level in the scalp tissue before treatment ($P<0.05$). β-catenin was differentially expressed by all three groups ($P<0.05$, one-way ANOVA). β-catenin expression in group 3 was significantly higher than that in group 1 and group 2 ($P<0.05$) but did not differ significantly between groups 1 and 2 at the observation endpoint (Figs. 4 and 5, Table 8).

LEF-1 expression in the scalp tissue at the observation endpoint was significantly increased in group 2 and group 3 ($P<0.05$), but decreased in group 1 ($P<0.05$) compared with that in the scalp tissue before treatment. LEF-1 was differentially expressed by all three groups ($P<0.05$, one-way ANOVA) with LEF-1 expression in group 3 being significantly higher than that in group 2 and group 1 ($P<0.05$). Additionally, LEF-1 expression in group 2 was significantly higher than

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that in group 1 at the observation endpoint ($P<0.05$, Figs. 4 and 5, Table 8).

Discussion

At present, several studies have investigated the use of microneedling to treat hair loss, and increasing evidence indicates that microneedling with percutaneous administration is an effective method for treating hair loss. Our team[16] previously used the same method to treat male AGA in 2017. Unlike the present study though, the previous study involved a treatment interval of 2 weeks and did not include molecular biology research. Dhurat *et al*.[17] used microneedling to treat 50 male AGA patients at an interval of 1 week, with 5% topical minoxidil twice daily; 50 patients in the control group were treated with minoxidil alone. After a 12-week treatment, the efficacy of the microneedling treatment was much better than that of the control treatment[17]. Harris and Murrel[18] used electrodynamic microneedling to introduce triamcinolone acetonide (IVTA) for treatment of two pediatric alopecia areata patients and found that the condition of both patients significantly improved. However, these experiments were clinical studies, and no further studies were performed at the gene or protein level. The present study is the first to verify the efficacy of microneedling treatment combined with topical minoxidil for treating AGA at the molecular level.

In the present clinical trial, changes in hair density and hair diameter in AGA patients from before to after treatment were observed through objective evaluation. The combination treatment group showed the best treatment efficacy. After treatment, hair loss at the top of the head of the volunteers was significantly improved, and the hair density was significantly increased. During the

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6 months of follow-up, only a small amount of regenerated hair was lost in most patients who received the combination treatment. During treatment, the treatment efficacy regarding hair loss at the top of the head was better than that for frontal hair loss, and with a shorter duration of hair loss, the treatment outcome improved. There were no significant differences in the densities of terminal hair, vellus hair, and total hair before and after the treatment between the microneedling only treatment group and the minoxidil only treatment group, while the difference in the average diameter of terminal hair between the two groups was significant. Treating AGA using topical minoxidil has been shown to significantly increase the terminal hair diameter[16], which is consistent with the results of the present study. The vellus hair density in each treatment group decreased after treatment, but the total hair density was significantly increased in groups 1 and 3. The reason may be that the treatment promoted growth from vellus hair to terminal hair.

The occurrence of adverse reactions among the three groups did not differ significantly, and the side effects did not require treatment and self-resolved within 3–4 days. The groups with microneedling treatment had a high incidence of scalp infection and lymphadenectasis. It is speculated that the enlarged lymph nodes in the neck or behind the ear were due to secondary infections caused by intense stimulation on the local scalp. Therefore, microneedling treatment should be applied under aseptic conditions.

Among the four branches of the Wnt signaling pathway, the classical Wnt/β-catenin pathway is the most closely related to the growth of hair follicles[19-21]. In the Wnt/β-catenin pathway, the genes that play a crucial role in the growth of hair follicles include β-catenin and LEF-1[22-24]. Wnt protein can increase the level of β-catenin in the cytoplasm by inhibiting the

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hydrolysis of β-catenin, and then a large amount of β-catenin is transported into the nucleus to bind with LEF-1, thereby activating the transcription of related target genes and promoting hair follicle stem cell proliferation and differentiation[22-24]. By inducing the expression of a mutant β-catenin gene, researchers created mice that lack β-catenin protein expression[25]. In the mouse embryonic stage, this gene mutation prevents the formation of hair follicle substrate[25]. After the formation of the hair follicle structure, the mutant β-catenin gene prevents hair follicles from entering the hair growth cycle[25]. In the absence of β-catenin protein expression, hair follicle stem cells cannot differentiate into follicular keratinocytes, thus confirming that β-catenin plays important roles in regulating the hair growth cycle and hair follicle stem cell differentiation[25]. LEF-1 is an essential factor of the Wnt signaling pathway. Previous studies showed that LEF-1 **is** expressed in the dermis of mouse follicles, suggesting that this gene is required for the growth of mouse hair follicles[26, 27]. Mutation of the LEF-1 gene can lead to the overexpression of LEF-1 or β-catenin and thus blockage of the β-catenin signaling pathway[26, 27]. The blockage negatively regulates the Wnt pathway, thereby inhibiting the differentiation of hair follicles and resulting in the formation of abnormal hair follicles[26, 27]. Therefore, the present study compared the expression of β-catenin and LEF-1 as target genes before and after treatment to assess treatment efficacy.

In the present study, the PCR array results suggested that the occurrence of AGA might be related to FZD3 expression. The encoded Frizzled protein FZD3 is a Frizzled transmembrane protein family member and also one of the receptor proteins of the Wnt signaling pathway[28, 29]. After the ligand Wnt5a binds to the FZD3 protein receptor on the cell membrane surface, it can

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activate the downstream signaling pathway[30, 31]. The function of the FZD3 protein is currently unclear and may be associated with follicular development, schizophrenia, and tumor formation[31]. Hung *et al*.[32] showed that FZD3 protein is only expressed in the epidermis and developing hair follicles in mouse skin. The results of PCR and in situ hybridization analysis identified FZD3 protein in the developing epidermis of the 13-day embryonic mice and the hair follicles of 15-day embryonic mice, indicating its function in hair follicle development[32]. In 17-day embryonic mice and 1-day neonatal mice (the hair follicles only appear in mice 3 days after birth), FZD3 expression is limited to epithelial keratinocytes[32]. In the skin of 7-day-old mice, FZD3 protein is expressed in the entire epidermis and outer cells of hair follicles[32]. Thus, FZD3 protein is closely associated with the development of hair follicles. Currently, the literature related to FZD3 expression and hair growth and development presents experimental research in animal models only, and no studies have been carried out in humans.

Microneedling treatment has a long history, but the application of microneedling treatment with drug administration for the treatment of hair loss has received widespread attention in recent years. A few clinical studies have been reported, but the underlying mechanism remains unclear. The RT-PCR results in this study showed that with minoxidil treatment alone, the expression levels of FZD3 and β-catenin were increased after treatment compared to those before treatment, and the differences were statistically significant, suggesting that topical minoxidil can activate the Wnt/β-catenin signaling pathway to promote hair growth. There were no differences in the expression levels of genes in the microneedling treatment alone group, and the experimental results were inconsistent with the clinical results, which may be related to the small sample size

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and low treatment frequency. In future studies, more accurate experimental results could be obtained by increasing the treatment depth, reducing the treatment cycle, and increasing the sample size. For the microneedling combined with topical minoxidil group, the expression levels of FZD3, β-catenin, and LEF-1 were significantly higher after treatment versus before treatment. These results indirectly demonstrate that topical minoxidil may treat hair loss by promoting the expression levels of genes closely related to hair growth. Meanwhile, it also suggests that microneedling treatment can improve the penetration of minoxidil.

The comparison of the western blotting results before and after treatment showed that the protein expression levels of FZD3, β-catenin, and LEF-1 were increased in the combination treatment group. Moreover, the expression levels of the proteins were significantly different among all groups. We found that the protein levels of the Wnt/β-catenin molecules studied here did not always reflect the mRNA levels as detected by qPCR, suggesting possible post-transcriptional or post-translational modifications are likely involved in the regulation of the Wnt/β-catenin pathway.

The qRT-PCR and western blotting results of the present study also showed that FZD3 expression is closely related to treatment efficacy. Therefore, this gene can be used as a new target for the treatment of male AGA in future in-depth studies. However, this study also had limitations. First, the sample size was small; the main reason was that the specimens were difficult to collect. Therefore, in future studies, sample collection should be strengthened to increase the sample size. Second, during the study, only patients in the combination group received scalp massage after the microneedling treatments to enhance absorption of the drug. This might be an influential factor

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for the therapeutic effect. In future studies, scalp massage should be done to all patients to avoid the potential impact on the therapeutic effect. Lastly, when conducting human experiments, there are certain limitations in the research methods. In the future, new experimental methods should be explored to more intuitively observe the changes in hair follicles and their surrounding tissues.

Conclusions

Microneedling combined with 5% minoxidil can be an effective treatment for AGA, and the mechanism may involve activation of the Wnt/β-catenin signaling pathway.

Acknowledgments

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Conflicts of interest

The authors declare that they have no competing financial interests.

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Tables

Table 1. Patient characteristics

	Group 1 (n = 23)	Group 2 (n = 23)	Group 3 (n = 25)
Age (years)	37.01± 8.43	38.42 ± 8.50	36.33 ± 8.04
Duration of hair loss (years)	7.74 ± 6.01	8.17 ± 4.61	8.03 ± 5.25
Non-vellus hair count/cm^2	96.76 ± 29.74	97.26 ± 32.70	94.27 ± 37.23
Vellus hair count/cm^2	50.21 ± 21.25	52.50 ± 22.04	53.78 ± 29.39
Total hair count/cm^2	146.97 ± 27.41	149.76 ± 40.91	148.05 ± 36.72
Mean hair thickness (μm)	67.84 ± 12.89	64.33 ± 17.87	65.23 ± 16.78
Hamilton-Norwood classification, n			
III	6	5	5
IV	7	8	8
V	8	7	9
VI	2	3	3
Previous treatments, n			
Never treated	16	16	18
Minoxidil	3	2	2
Finasteride	2	4	2
Traditional Chinese medicine	2	1	3

Data are presented as the mean ± standard deviation. Group 1: 5% minoxidil; Group 2: microneedling; and Group 3: microneedling combined with 5% minoxidil.

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Table 2. Changes in hair density after 24 weeks of treatment

Hair count/cm^2	One-sample *t* test			One-way ANOVA	P value Two-sample *t* test		
	Group 1 (n = 23)	Group 2 (n = 23)	Group 3 (n = 25)	P value among the three groups	Group 1 vs Group 2	Group 1 vs Group 3	Group 2 vs Group 3
Non-vellus hair count	28.01 ± 7.42 P = 0.002	32.09 ± 4.51 P = 0.004	56.45 ± 7.82 P = 0.002	P < 0.001	P = 0.513	P < 0.001	P < 0.001
Vellus hair count	-13.74 ± 3.41 P = 0.014	-10.56 ± 2.83 P = 0.064	-16.67 ± 6.48 P = 0.021	P = 0.578	P = 0.592	P = 0.617	P = 0.297
Total hair count	14.27 ± 3.24 P = 0.046	21.53 ± 6.19 P = 0.074	39.78 ± 7.79 P < 0.001	P < 0.001	P = 0.190	P < 0.001	P = 0.007

Data are presented as the mean ± standard deviation. Group 1: 5% minoxidil; Group 2: microneedling; and Group 3: microneedling combined with 5% minoxidil.

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Table 3. Mean change in hair thickness from baseline to week 24

	One-sample *t* test			One-way ANOVA	P value Two-sample *t* test		
	Group 1 (n = 23)	Group 2 (n = 23)	Group 3 (n = 25)	P value among the three groups	Group 1 vs Group 2	Group 1 vs Group 3	Group 2 vs Group 3
Change in Non-vellus hair thickness (μm)	18.70 ± 6.17 P < 0.001	10.41 ± 4.83 P = 0.058	22.46 ± 3.87 P < 0.001	P = 0.002	P = 0.015	P = 0.259	P < 0.001

Data are presented as the mean ± standard deviation. Group 1: 5% minoxidil; Group 2: microneedling; and Group 3: microneedling combined with 5% minoxidil.

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Table 4. Fold changes in the expression of genes involved in the Wnt/β-catenin pathway at sites of hair loss (n=3) and non-hair loss (n=3) scalps by PCR array analysis

3D Profile	A	B	C	D	E	F	G
1	AES	CTNNBIP1	FGF4	FZD8	NFATC1	SFRP4	WNT2
	-1.04	-1.31	1.13	1.71	1.07	-1.12	1.09
2	APC	CXXC4	FOSL1	FZD9	NKD1	SOX17	WNT2B
	-1.03	-1.13	1.97	-1.36	1.00	-1.15	1.36
3	AXIN1	DAAM1	FOXN1	GSK3A	NLK	TCF7	WNT3
	1.03	-1.01	2.18	1.04	1.09	1.91	1.81
4	AXIN2	DAB2	FRAT1	GSK3B	PITX2	TCF7L1	WNT3A
	-1.04	-1.03	1.26	1.19	-1.83	1.34	1.10
5	BCL9	DIXDC1	FRZB	JUN	PORCN	TLE1	WNT4
	-1.03	-1.00	1.42	1.24	-1.13	1.34	1.07
6	BTRC	DKK1	FZD1	KREMEN1	PPARD	VANGL2	WNT5A
	1.22	1.64	1.39	-1.19	-1.03	1.45	1.64
7	CCND1	DKK3	FZD2	LEF1	PRICKLE1	WIF1	WNT5B
	1.41	1.01	-1.36	2.52	1.73	1.83	1.22
8	CCND2	DVL1	FZD3	LRP5	PYGO1	WISP1	WNT6
	1.54	1.16	2.45	1.17	1.13	-1.58	1.03
9	CSNK1A1	DVL2	FZD4	LRP6	RHOA	WNT1	WNT7A
	1.06	1.07	-1.28	1.87	1.01	1.03	1.32
10	CSNK2A1	EP300	FZD5	MAPK8	RHOU	WNT10A	WNT7B
	1.02	1.06	1.15	1.01	1.03	1.20	1.18
11	CTBP1	FBXW11	FZD6	MMP7	RUVBL1	WNT11	WNT8A
	1.11	1.09	1.34	1.27	1.13	1.37	1.05
12	CTNNB1	FBXW4	FZD7	MYC	SFRP1	WNT16	WNT9A
	1.66	1.02	1.80	1.25	3.28	-1.13	1.25

Data represent the fold change in expression. Values highlighted in color indicate molecules with significant differences (fold change values >1.5 considered significant up-regulation and <-1.5 considered significant down-regulation) in expression. Red represents upregulated, blue represents downregulated, and FZD3 is in position C8.

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Table 5. Fold changes in expression of genes of the Wnt/β-catenin pathway at hair loss sites from baseline (n=3) to after treatment (n=3) by PCR array analysis

3D Profile	A	B	C	D	E	F	G
1	AES	CTNNBIP1	FGF4	FZD8	NFATC1	SFRP4	WNT2
	-1.25	-1.42	-1.12	1.10	1.25	2.87	-3.63
2	APC	CXXC4	FOSL1	FZD9	NKD1	SOX17	WNT2B
	1.17	1.05	3.64	-1.75	1.02	-1.03	-1.02
3	AXIN1	DAAM1	FOXN1	GSK3A	NLK	TCF7	WNT3
	-1.06	-1.03	1.28	-1.16	1.27	1.20	1.11
4	AXIN2	DAB2	FRAT1	GSK3B	PITX2	TCF7L1	WNT3A
	-1.50	1.82	1.13	-1.15	-1.48	1.30	-1.11
5	BCL9	DIXDC1	FRZB	JUN	PORCN	TLE1	WNT4
	-1.50	-1.51	1.07	-1.08	-1.22	-1.02	-1.05
6	BTRC	DKK1	FZD1	KREMEN1	PPARD	VANGL2	WNT5A
	-1.06	1.59	-1.29	-1.22	-1.21	1.22	1.31
7	CCND1	DKK3	FZD2	LEF1	PRICKLE1	WIF1	WNT5B
	-1.06	-1.03	1.24	1.23	1.11	1.44	-1.23
8	CCND2	DVL1	FZD3	LRP5	PYGO1	WISP1	WNT6
	1.19	-1.21	1.85	-1.01	-1.04	1.31	1.02
9	CSNK1A1	DVL2	FZD4	LRP6	RHOA	WNT1	WNT7A
	-1.13	1.03	-1.06	1.45	-1.02	3.06	1.60
10	CSNK2A1	EP300	FZD5	MAPK8	RHOU	WNT10A	WNT7B
	-1.02	-1.19	-1.33	-1.01	1.03	1.04	1.38
11	CTBP1	FBXW11	FZD6	MMP7	RUVBL1	WNT11	WNT8A
	-1.22	-1.01	1.21	-1.07	-1.16	1.22	1.32
12	CTNNB1	FBXW4	FZD7	MYC	SFRP1	WNT16	WNT9A
	1.19	-1.10	-1.05	1.07	1.85	1.39	1.38

Data represent the fold change in expression. Values highlighted in color indicate molecules with significant differences (fold change values >1.5 considered significant up-regulation and <-1.5 considered significant down-regulation) in expression. Red represents upregulated, blue represents downregulated, and FZD3 is in position C8.

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Table 6. Comparison of FZD3 mRNA expression at scalp sites with hair loss vs. without hair loss, as measured by qRT-PCR

	Hair loss scalp (n=6)	Normal scalp (n=6)
FZD3[*]	1.69 ± 0.66	8.97 ± 1.47

Data are presented as the mean ± standard deviation. [*]P<0.05

Table 7. Changes in FZD3, β-catenin, and LEF-1 mRNA expression levels after 24 weeks of treatment compared with baseline, as determined by qRT-PCR

	FZD3	β-catenin	LEF-1
Group 1 (n=9)	1.28 ± 0.33[Δ]	23.57 ± 3.36[Δ]	0.12 ± 0.03
Group 2 (n=9)	-0.66 ± 0.24	-1.40 ± 0.54	2.08 ± 0.52[*]
Group 3 (n=9)	4.94 ± 1.26[*#Δ]	2.78 ± 0.06[*#Δ]	6.20 ± 1.16[*#Δ]

Data are presented as the mean ± standard deviation. Group 1: 5% minoxidil; Group 2: microneedling; and Group 3: microneedling combined with 5% minoxidil. [*]P<0.05 compared with group 1. [#]P<0.05 compared with group 2. [Δ]P<0.05 after 24 weeks of treatment compared with baseline.

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Table 8. Changes in protein expression levels of FZD3, β-catenin, and LEF-1 after 24 weeks of treatment compared with baseline, as determined by western blotting

	FZD3	LEF-1	β-catenin
Group 1 (n=9)	-0.74 ± 0.16	-1.42 ± 0.31$^{\Delta}$	-0.69 ± 0.15
Group 2 (n=9)	1.04 ± 0.17$^{*\Delta}$	1.13 ± 0.07$^{*\Delta}$	0.76 ± 0.24
Group 3 (n=9)	3.02 ± 1.13$^{*\#\Delta}$	2.39 ± 0.64$^{*\#\Delta}$	2.33 ± 0.62$^{*\#\Delta}$

Data are presented as the mean ± standard deviation. Group 1: 5% minoxidil; Group 2: microneedling; and Group 3: microneedling combined with 5% minoxidil. *P<0.05 compared with group 1. $^{\#}$P<0.05 compared with group 2. $^{\Delta}$P<0.05 after 24 weeks of treatment compared with baseline.

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Figure legends

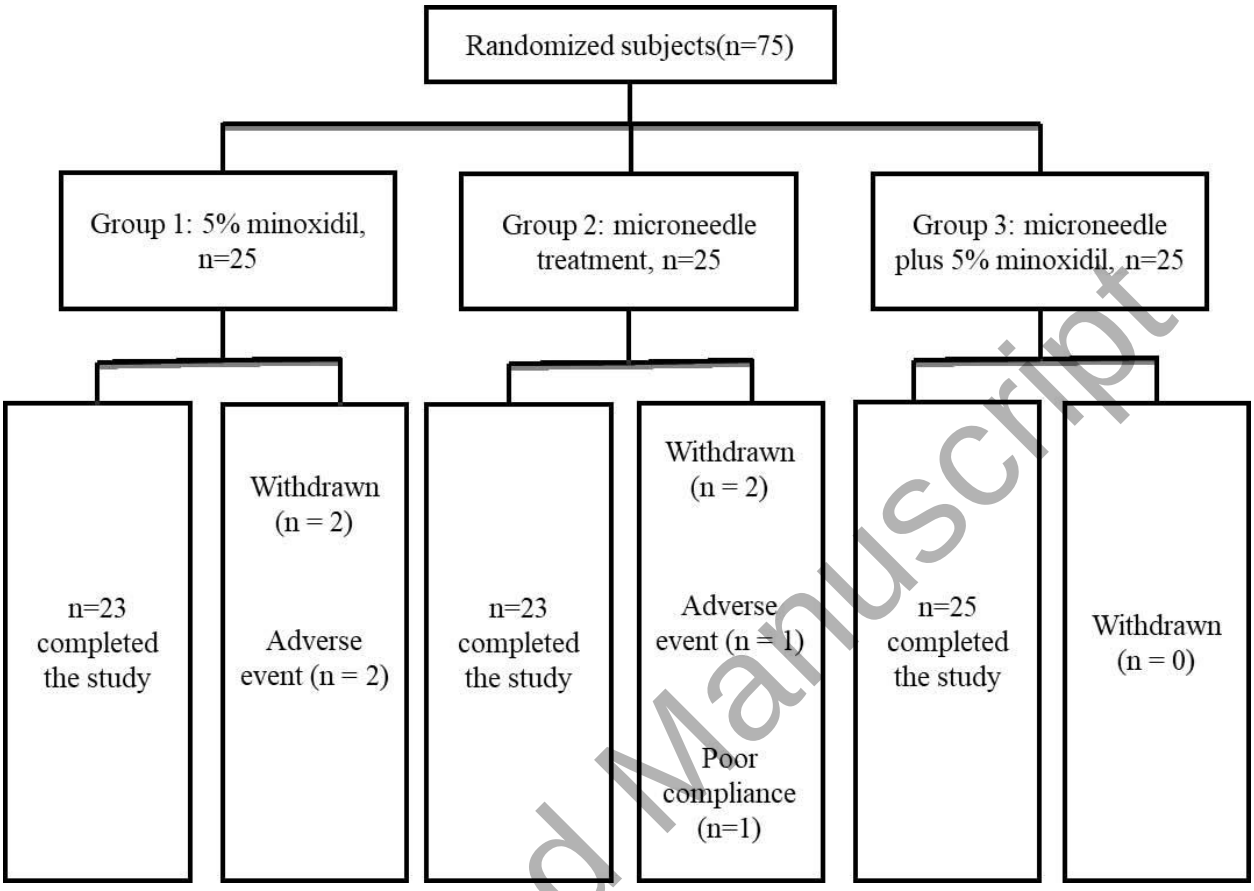

Fig. 1. Randomized controlled trial design.

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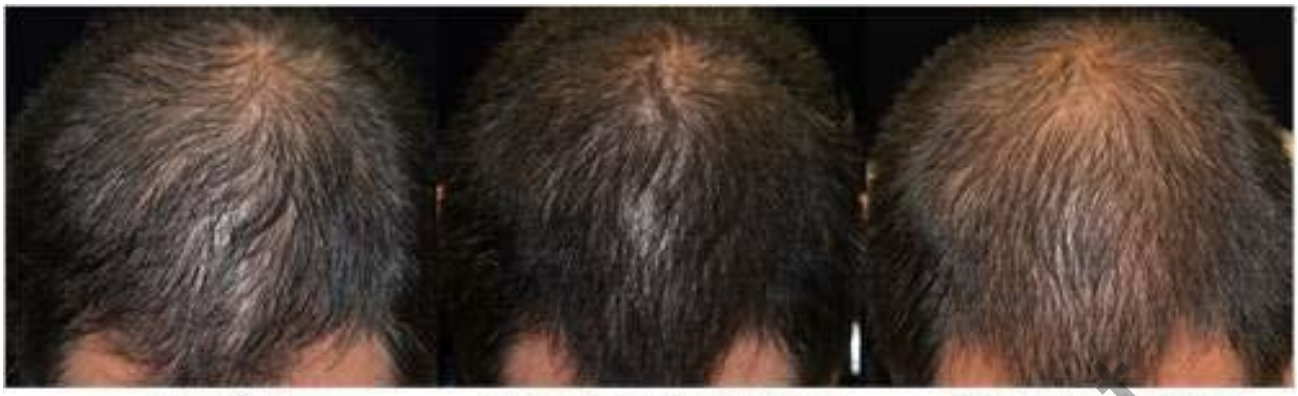
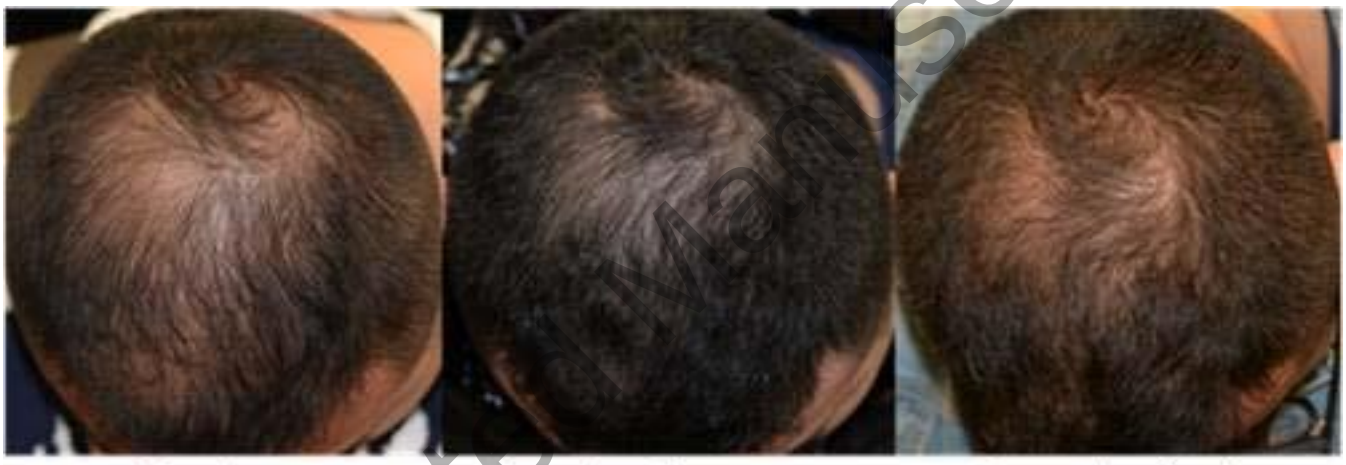


Baseline | 24 weeks of treatments | 6 months after the 24 weeks of treatments



Baseline | 24 weeks of treatments | 6 months after the 24 weeks of treatments

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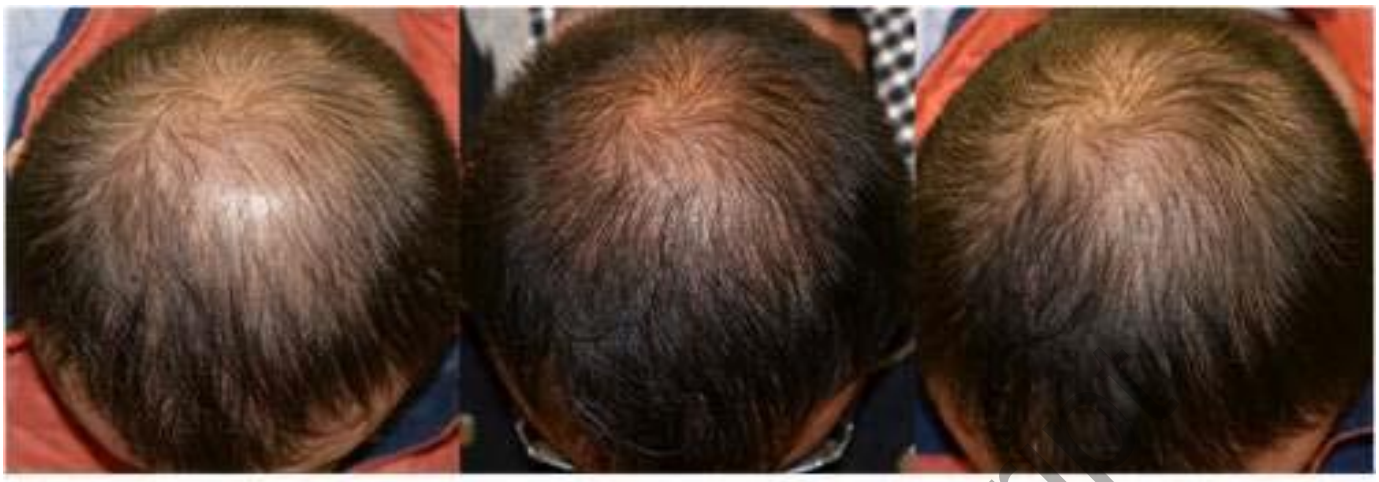

Fig. 2. Photographs of patients at baseline, after 24 weeks of treatment, and 6 months after the end of treatment. (a) Group 1, (b) Group 2, and (c) Group 3. Group 1: 5% minoxidil; Group 2: microneedling; and Group 3: microneedling combined with 5% minoxidil.

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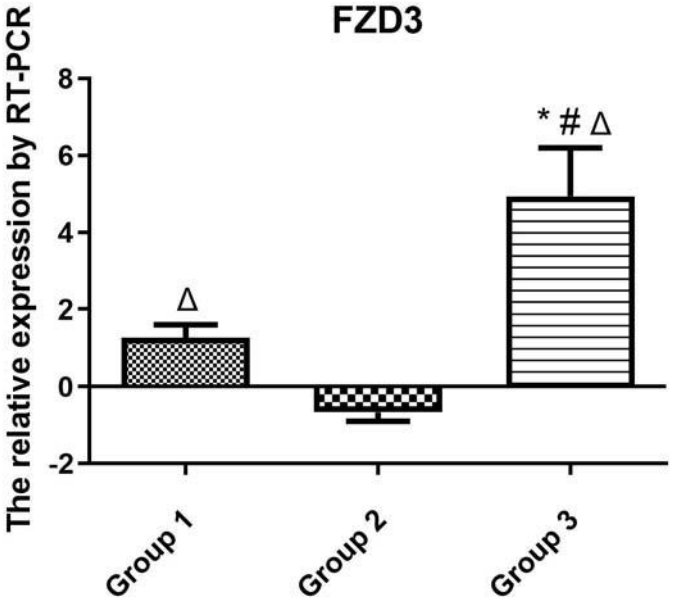

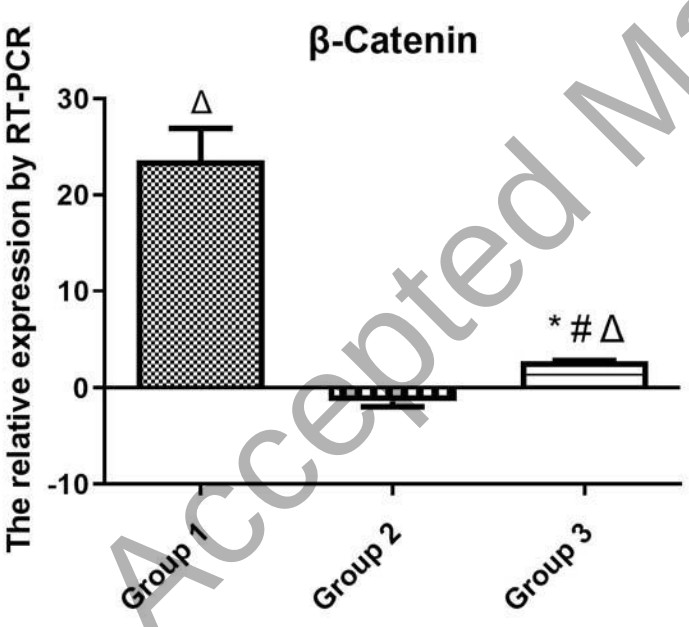

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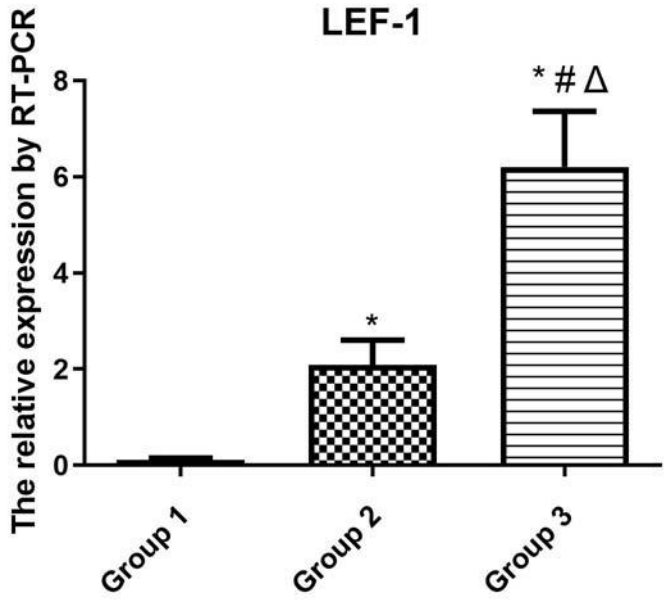

Fig. 3. Changes in the mRNA expression levels of FZD3, β-catenin, and LEF-1 after 24 weeks of treatment compared with baseline, as determined by qRT-PCR. (a) FZD3, (b) β-catenin, and (c) LEF-1. Group 1 (n=9): 5% minoxidil; Group 2 (n=9): microneedling; and Group 3 (n=9): microneedling combined with 5% minoxidil. $^{*}P<0.05$ compared with group 1. $^{\#}P<0.05$ compared with group 2. $^{\Delta}P<0.05$ after 24 weeks of treatment compared with baseline.

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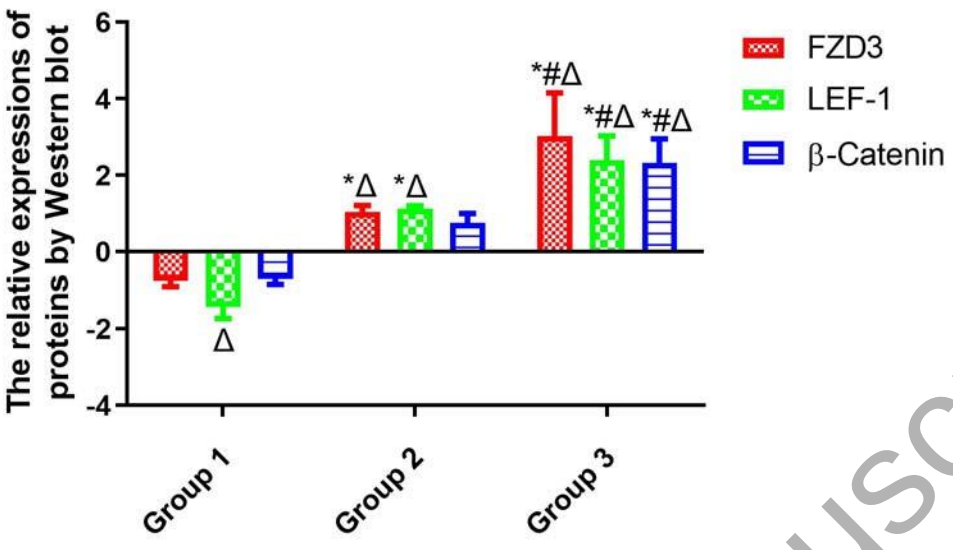

Fig. 4. Changes in the protein expression levels of FZD3, β-catenin, and LEF-1 after 24 weeks of treatment compared with baseline, as measured by western blotting. Group 1 (n=9): 5% minoxidil; Group 2 (n=9): microneedling; and Group 3 (n=9): microneedling combined with 5% minoxidil. $^{*}P<0.05$ compared with group 1. $^{\#}P<0.05$ compared with group 2. $^{\Delta}P<0.05$ after 24 weeks of treatments compared with baseline.

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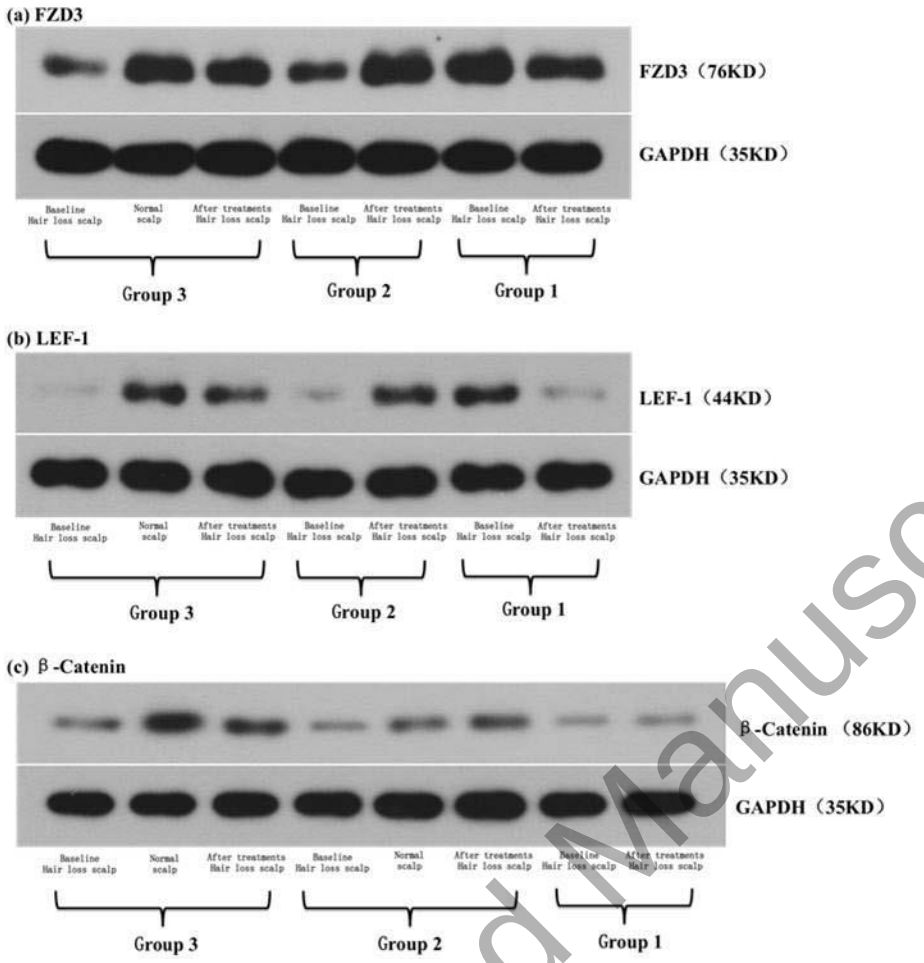

Fig. 5. Changes in the protein expression levels of FZD3, β-catenin, and LEF-1 after 24 weeks of treatment compared with baseline, as measured by western blotting. Group 1 (n=9): 5% minoxidil; Group 2 (n=9): microneedling; and Group 3 (n=9): microneedling combined with 5% minoxidil.

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