Original article

The Success of Thread-embedding Therapy in Generating Hair Re-growth in Mice Points to Its Possibly Having a Similar Effect in Humans

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Abstract

Objectives: Recently, thread-embedding therapy (TET) has been widely applied in Korean medicine for cosmetic purposes such as reducing skin wrinkles. An inserted thread was reported to have induced continuous stimulation, followed by support for connective tissue regeneration. However, the potential role of TET in hair-growth has not yet been reported.

Methods: We designed this study to evaluate whether TET has a hair-growth-promoting effect. C57 black 6 (C57BL/6) mice were divided into three groups: normal saline-treated, minoxidil-treated, and thread-embedded groups. Normal saline or 5% minoxidil was topically sprayed on the dorsal skin of the mice once a day for 16 days. Medical threads were embedded into the dorsal skin of the mice in a single application. Hair growth activity was evaluated by using dermoscopic and microscopic observations. Sections of the dorsal skin were stained with hematoxylin and eosin. Expressions of bromodeoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA), fibroblast growth factor-7 (FGF-7), and fibroblast growth factor-5 (FGF-5) were detected by using immunohistochemical staining. A reverse transcription-polymerase chain reaction (RT-PCR) analysis was adopted to measure the messenger RNA (mRNA) expressions of FGF-7 and FGF-5.

Results: TET enhanced anagen development in the hair follicles of C57BL/6 mice. The expressions of BrdU and PCNA, both of which imply active cellular proliferation, were increased by using TET. Moreover, TET increased the expression of FGF-7, an anagen-inducing growth factor, while decreasing the expression of FGF-5, an anagen-cessation growth factor, both at the protein and the mRNA levels.

Conclusion: TET enhanced hair re-growth in C57BL/6 mice. TET regulated the expressions of anagen-associated growth factors and activated the proliferation of hair follicular cells in depilated skin lesions. Considering its long-lasting effect, TET may be a good alternative therapeutic for the treatment of alopecia.

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1. Introduction

An increasing number of people suffer from hair loss. Although it is not a life-threatening problem, hair loss can cause serious psychological distress [1]. Various causes of hair loss have been described, including hormonal, genetic, stress-induced, and autoimmune disease-related causes [2-6]. However, the underlying mechanisms leading to baldness are poorly understood owing to the complexity of the molecular signals that orchestrate hair growth [7].

Although many studies have been conducted to develop drugs that prevent or reverse hair loss, only finasteride and minoxidil have been approved by the Food and Drug Administration of the United States (FDA, U.S.A.) for promoting hair growth [8]. However, the effectiveness of these drugs varies greatly among individuals; moreover, the drugs have unwanted side effects [9-13]. Therefore, a demand for other safe and effective treatments for hair loss continues to exist.

Thread-embedding therapy (TET), a type of dermal needle therapy, is defined as inserting thread into skin lesions for the purpose of prolonging therapeutic stimulation. In East Asia, including Korea and China, TET has been used to treat various chronic diseases owing to its long-lasting effects [14-17]. Recently, TET has been widely used for cosmetic purposes, such as reducing skin wrinkles and tightening facial skin, based on the belief that TET will help to regenerate connective tissue [18, 19]. However, no effect of TET on hair growth has yet to be reported.

We hypothesized that TET could enhance anagen induction in the hair follicles of skin connective tissue. Thus, in this study, we evaluated the hair re-growth-promoting effects of TET.

2. Materials and Methods

Polydioxanone medical thread-embedding needles (Miracu) were purchased from Dongbang Acupuncture (Boryeong, Korea).

Seven-week-old male C57 black 6 (C57BL/6) mice were purchased from Samtaco Bio Korea, Ltd. (Osan, Korea) and were allowed to adapt to their new environment for 1 week. The mice were housed in certified standard laboratory cages and provided with food and water *ad libitum* prior to the experiment. A total of 15 mice were assigned to three groups (5 mice in each group): the normal saline-sprayed, minoxidil-sprayed, and thread-embedded groups. The animal protocol used in this study was reviewed and approved by Pusan National University's Institutional Animal Care and Use Committee (PNU–IACUC) in accordance with established ethical procedures and scientific care (approval number: PNU-2014-0581).

As described in a previous study, the dorsal hair of 8-week-old C57BL/6 mice, whose hair follicles were in the telogen phase of the hair growth cycle, were depilated to induce homogeneous anagen induction [20]. One day after removal of the dorsal hair, the mice in the negative and the positive control groups were treated with normal saline or 5% minoxidil, respectively, once a day for 16 days. Mice in the third group received TET 1 day after removal of the dorsal hair (Fig. 1). Fifteen threads (1 cm in length) were embedded in the dorsal skin of each mouse (1 cm^2). The hair growth and thickness at the sites of the dorsal skin lesions in the C57BL/6 mice was measured by using dermoscopy (Sometech, Inc., Seoul, Korea).

The C57BL/6 mice were euthanized 16 days after the first treatment. Dorsal skin samples were fixed for 24 hours at room temperature in bouin's solution and were then embedded in paraffin. Sections of tissue, 7-μm thick, were cut and mounted on glass slides, deparaffinizedin xylene, and processed for hematoxylin and eosin staining. Processed skin tissues were examined under light microscopy.

Bromodeoxyuridine (BrdU), which is used to label proliferating cells in tissue sections [21], was intraperitoneally injected at a dose of 50 μg/g of body weight (BW) twice per day for three consecutive days after hair removal, as described in a previous study [22]. Dorsal skin tissues were collected on the 16 days after the first treatment and were subjected to immunostaining for BrdU.

The permeated tissue sections were incubated with a selected antibody according to the manufacturer's instructions: mouse anti- BrdU antibody (1 : 200, Santa Cruz, CA, U.S.A.) for the detection of BrdU, proliferating cell nuclear antigen antibody (1 : 1000, Santa Cruz, CA, U.S.A.) for the detection of PCNA, fibroblast growth factor-7 (FGF-7) antibody (1 : 200, Santa Cruz, CA, U.S.A.) for the detec-
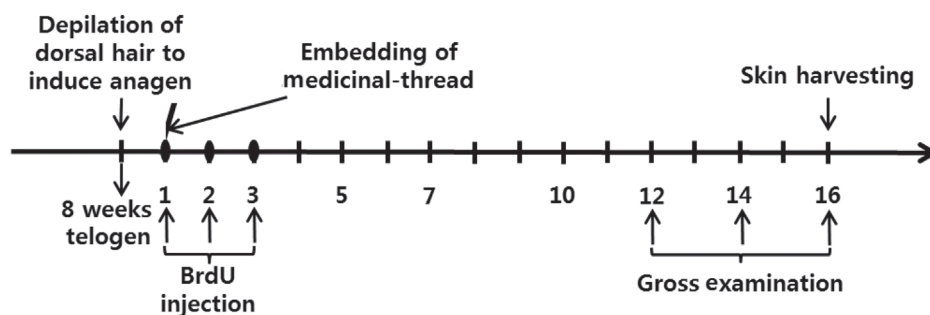

Figure 1 Scheme of the experiment.

C57BL/6, C57 black 6; BrdU, Bromodeoxyuridine; ●, BrdU injection; /, medical thread embedding.
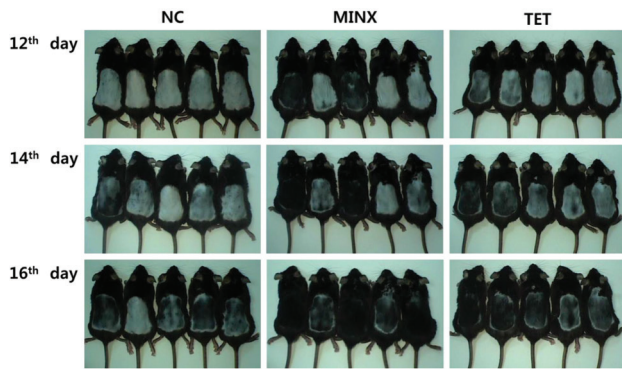
Figure 2 Effects of TET on hair re-growth in C57BL/6 mice.

The dorsal skin of 8-week-old male C57BL/6 mice was depilated to synchronize anagen induction. Control groups received topical applications of normal saline or 5% minoxidil for 16 consecutive days. The experimental group of mice received TET in a single application. The dorsal skin of the mice in each group was photographed on days 12, 14, and 16 after depilation. Hair re-growth, as well as darkness of skin, was increased in mice treated with TET when compared with the control mice.

NC, normal control; MINX, minoxidil; TET, thread-embedding therapy; C57BL/6, C57 black 6.

tion of FGF-7, or fibroblast growth factor-5 (FGF-5) antibody (1 : 200, Santa Cruz, CA, U.S.A.) for the detection of FGF-5. Bound antibodies were sequentially reacted with biotinylated goat anti-mouse immunoglobulin G (IgG) and avidin-biotinylated peroxidase complex (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 30 minutes in a moisture chamber. Immunoreactivity was visualized by using diaminobenzidine.

Total ribonucleic acid (RNA) was isolated by using TRIzol reagent (Invitrogen) and was converted to complementary deoxyribonucleic acid (cDNA) by using AccuPower RT PreMix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Specific DNA sequences were amplified with AccuPower PCR PreMix (Bioneer, Daejeon, Korea). The oligonucleotide primer sequences were as follows: FGF-7, forward 5'-AGATCATGCTTCCACCTCGT-3'and reverse 5'-TGGGTCCCTTTCACTTTGCC-3'; FGF-5, forward 5'-ACCCGGATGGCAAAGTCAAT-3' and reverse 5'-TGGT-TTCACCGGTGGCTTTT-3'; GAPDH, forward 5'-GGAGC-CAAAAGGGTCATCAT-3' and reverse 5'-GTGATGGCAT-GGACTGTGGT-3'. Amplified products were analyzed in 1.0% agarose gel under ultraviolet light, and the images-were captured using the GelDoc-It TS Imaging System (UVP, LLC, Upland, CA, U.S.A.).

Data were expressed as means ± standard deviations (SDs). Statistical differences between means were determined by using the one-way analysis of variance (ANOVA) for repeated measures. *P*-values less than 0.05 were considered significant.

The dorsal hairs of 8-week-old C57BL/6 mice in the telogen stage of the hair growth cycle were depilated to synchronize anagen induction. We assigned the mice into the following 3 groups (5 mice per group): group 1, normal sa-line-applied negative control; group 2, 5% minoxidil-treated positive control; group 3, medical thread-embedded experimental group. One day after depilation of the dorsal hair, the dorsal skin of the mice was topically sprayed with normal saline or 5% minoxidil or was embedded with medical thread. BrdU was administered twice a day for three consecutive days after hair removal. The dorsal skin was collected on the 16th day after treatment and was subjected to hematoxylin and eosin staining.

3. Results

Fig. 2 presents gross images of hair re-growth on days 12, 14, and 16 after the depilation. The TET-induced prominent hair re-growth was comparable to that obtained by treatment with minoxidil. The thickness of the hair thickness was also greater in the thread-embedded group than in the normal saline applied group (Fig. 3).

Histological analysis showed that TET enhanced anagen induction (Fig. 4(A, B, C)). In the normal saline-applied group, hair follicles resided in the dermis, and the inner root sheath was absent, indicating that the hair follicles had not reached the mature anagen stage (Fig. 4(A)). On the other hand, the hair follicles of mice in the minoxidil and the TET groups exhibited emergence of hair shafts through the epidermis, indicating full development to the anagen VI phase (Fig. 4(B, C)). Immunohistochemical analysis confirmed that the expressions of BrdU and proliferating cell nuclear antigen (PCNA) were prominently increased by using TET (Fig. 4(F, I)). A strongly positive reaction against BrdU was observed at the bulge, the inner and the outer root sheath, and the panniculus carnosus, indicating that TET had induced active cell division in the hair follicle. TET also increased the expression of PCNA at the hair bulb (Fig. 4(I), arrow).

In the TET group, a strong expression of FGF-7 was observed at the outer root sheath. However, the expression of FGF-5 was prominently decreased in the TET group (Fig. 5(A)). reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the expression of FGF-7 messenger RNA (mRNA) was higher in the TET group than in the NC group whereas the expression of FGF-5 mRNA was lower in the TET group (Fig. 5(B)).

4. Discussion

TET refers to the subcutaneous insertion of surgical thread at acupoints or skin. It is an improved form of needle-embedding therapy, which involves insertion of fine metal fragments into subcutaneous tissue in order to increase needle sensation and prolong the duration of stimulation [23]. Needle-embedding therapy is associated with serious complications such as needle migration and penetration of solid organs [20, 24]. Replacing embedded needles with absorbable materials such as catgut eliminates the danger of needle retention.

Embedded threads are believed to have a constant mechanical or chemical stimulatory effect on the subcuta-
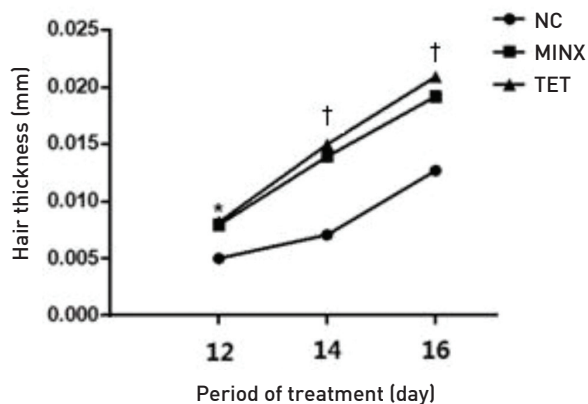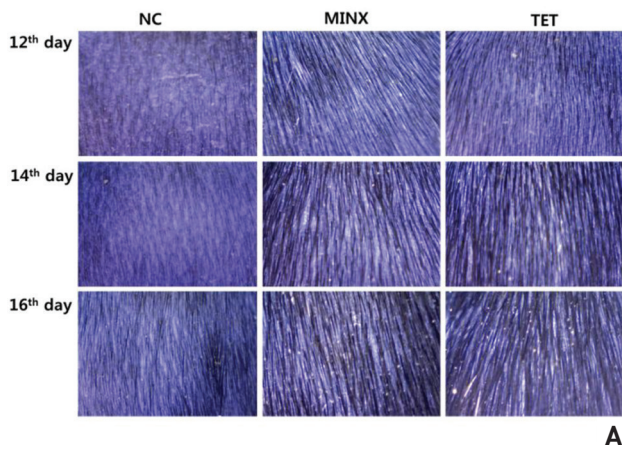
A

B

Figure 4 Effects of TET on anagen induction and cellular proliferation in the depilated dorsal skin of C57BL/6 mice.

(A, B, C) Sixteen days after depilation, the dorsal skin from the C57BL/6 mice was collected and examined after staining with hematoxylin and eosin. (C) TET promoted anagen induction in the hair follicle. (F, I) Immunohistochemical analysis reveals a TET-induced proliferation of cells in the hair follicle. (I) A strong reaction against PCNA was observed in the hair bulb (arrow). The original magnification was ×100.
NC, normal control; MINX, minoxidil; TET, thread-embedding therapy; C57BL/6, C57 black 6, PCNA, proliferating cell nuclear antigen.

Figure 3 TET also increased the hair thickness of depilated skin lesions on C57BL/6 mice.

(A) The dorsal skin of the mice in the control and the experimental groups was photographed at 12, 14 and 16 days after hair depilation. (B) Compared with the normal saline-applied control group, the TET-treated group had significantly increased hair thickness. Values are means ± SDs. $^*P < 0.01$ compared to the control.
NC, normal control; MINX, minoxidil; TET, thread-embedding therapy; C57BL/6, C57 black 6, SDs, standard deviations.

neous tissues beneath acupoints or skin. Therefore, TET has been widely used to treat chronic diseases [14-17]. Recently, TET has received great attention as a cosmetic procedure in the belief that it has potential to promote connective-tissue regeneration. TET has been reported to facilitate collagen synthesis, thereby lifting facial tissues and improving skin elasticity and moisture [18, 19]. Although no studies of TET's effect on hair growth have been reported, we considered it is feasible that the regenerative potential of TET on connective tissue could also influence the hair growth cycle.

The hair follicle undergoes successive cyclic changes. The anagen phase starts with the first emergence of a new hair follicle in the dermis. Hair matrix cells, which are located in the inferior segment of the hair follicle, continuously proliferate and differentiate to form the hair shaft. Elon-

gation and thickening of the hair shaft occurs only in the anagen phase. Following the catagen phase leading to involution of the hair follicle, the proliferative activity of the cells in the hair matrix stops, and cell death occurs in the follicular keratinocyte. In the telogen phase, all activities in the hair follicle cease, and thick pigmented hair changes to inactive dead hair or club hair. Finally, the club hair is ejected, and a new hair emerges from the follicle, signaling the start of a new anagen phase [25-27]. Hair miniaturization, which is the characteristic feature of androgenic alopecia (AGA), is associated with shortening of the anagen phase [28]. Therefore, drugs that extend the anagen phase can prevent hair miniaturization and are good candidate therapies for AGA.
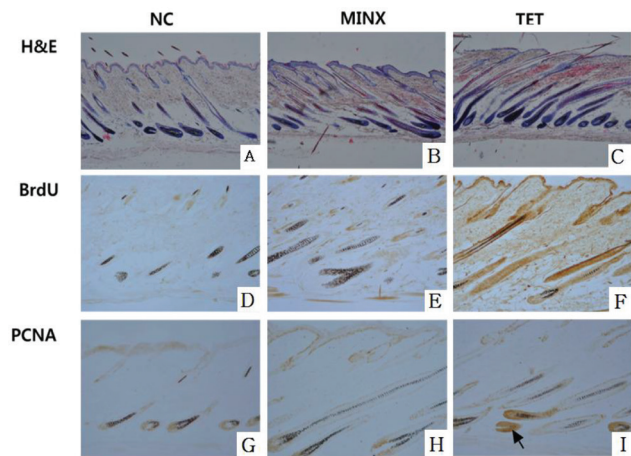
In this study, we explored the hair growth-promoting effects of TET, mainly focusing on the capability of anagen induction. Our results showed that TET increased the hair's thickness and hair re-growth in C57BL/6 mice by promoting anagen induction and cell proliferation at the hair bulb (Figs. 3, 4). Histologic studies confirmed that the hair follicles in the TET-treated mice were fully developed to the anagen VI phase (Fig. 4(C)), the same level of follicular development found in the minoxidil treated-group (Fig. 4(B)). TET significantly increased the expressions of BrdU and PCNA, the markers of cell proliferation in living tissue (Fig. 4(F, I)). TET had an especially strong effect on increasing the level of PCNA at the hair bulb lesion (Fig. 4(I), arrow), suggesting that TET enhances the division of
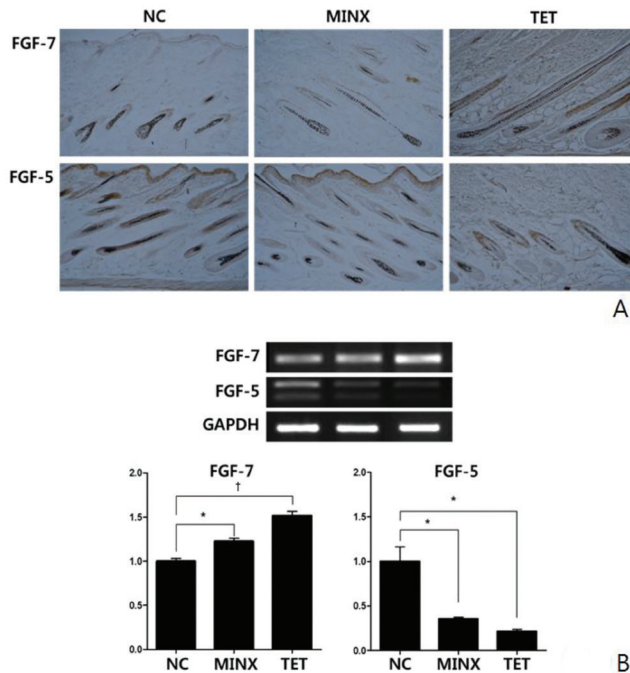
Figure 5 Induction by TET of growth factors that play important roles in hair follicle development.

Dorsal skin was collected from C57BL/6 mice treated with TET for 16 days. (A) The protein expressions of FGF-7 and FGF-5 were analyzed by using an immunohistochemical assay. (B) The mRNA expressions of FGF-7 and FGF-5 were analyzed by using the RT-PCR. The relative levels of FGF-7 and FGF-5 mRNA are shown as means ± SDs for three experiments. $^*P < 0.05$ and $^†P < 0.01$ compared to the control.
NC, normal control; MINX, minoxidil; TET, thread-embedding therapy; FGF-7, fibroblast growth factor-7; FGF-5, fibroblast growth factor-5; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; C57BL/6, C57 black 6; mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; SDs, standard deviations.

cells in the hair matrix, thereby increasing the hair's thickness during the anagen phase. Hair shaft synthesis in the anagen phase is regulated by a number of interactions between dermal papilla and the overlying follicular epithelium [29]. Numerous growth factors and molecules are involved in the formation of the hair shaft. Among them, FGF-7 has been known to play an important role in the anagen development of hair follicles [30-32]. On the other hand, FGF-5 has been reported to inhibit hair elongation by blocking the activation of dermal papilla cells [29, 33]. TET increased the expression of FGF-7 at the outer root sheath (Fig. 5(A), upper third column). The strong expression of FGF-5 that was observed at the inner and outer root sheath and the epidermal keratinocytes of the NC group was decreased by using TET (Fig. 5(A), lower first & third columns). The results of the RT-PCR analysis were consistent with the results of the immunohistochemical assay (Fig. 5(B)).

5. Conclusion

Taken together, our results show that TET enhances anagen induction in the depilated dorsal skin of C57BL/6 mice by up-regulating hair follicular cell proliferation and controlling the expressions of FGF-7 and FGF-5, which are involved in anagen induction and cessation, respectively. Interestingly, a single application of TET resulted in hair growth comparable to that achieved with consecutive applications of minoxidil. Considering its long-lasting and proliferating potentials, TET can be considered to be a good alternative therapeutic for the treatment of patients with alopecia.

Acknowledgements

Conflict of interest

The authors declare that there are no conflict of interest.

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