Effects of CO$_2$ fractional laser on hair growth in C57BL/6 mice and potential underlying mechanisms

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To the Editor: Hair loss or alopecia is a very common disease, which has the potential to impact the quality of life, physical attractiveness, and interpersonal relationships. In recent years, lasers and light therapy have been used as alternative or supplementary therapy for hair loss. In 2007, low-level laser therapy was approved by the United States Food and Drug Administration as a safe and effective treatment on hair loss.[1] The non-ablative 1550 nm fractional laser also appears to be useful for increasing hair density in patients with androgenetic alopecia.[2] Moreover, our previous study found that ablative CO$_2$ fractional laser therapy in combination with hair growth factors proves more effective for the treatment of patients with androgenetic alopecia than topical hair growth factors alone.[3] However, the detailed mechanisms underlying hair re-growth and injury induced by the laser remains unclear. In the present study, we assessed the expression of several inflammatory cytokines related to wound healing and Wnt signal pathway molecules at different time points before and after CO$_2$ fractional laser therapy. Potential mechanisms behind fractional CO$_2$ laser-induced hair growth were then explored in mice.

All experiments were performed according to protocols approved by the Animal Studies Subcommittee of Capital Medical University. Six-week-old female C57BL/6 mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) were housed for 1 week and then anaesthetized with 0.04 mL/10 g 10% chloral hydrate through intra-peritoneal injection. Mouse dorsal hair was carefully shaved with an electrical hair clipper without injury to the skin. Skin color change from pink to black indicated transition from telogen to anagen.[4] To determine the proper dose parameters, the shaved backs of 12 mice were irradiated with a 10,600 nm CO$_2$ fractional laser (Pixel CO$_2$; Alma Lasers Ltd., Aesthetic Mode, Israel). The dorsal skin of each mouse divided into four areas and each area treated with one of four energy settings (6, 12, 18, and 24 mJ/spot, respectively) and one

beam density (361 spots/cm^2) in a single pass [Supplementary Figure 1, http://links.lww.com/CM9/A30]. For parameter selection and immediate microscopic inspection of histologic response, each irradiation area was 1 cm^2 in size and spaced at an interval distance of 1 cm.

To explore potential mechanisms of hair re-growth, the remaining 48 mice were treated on one of three divided areas at the dose determined by parameter assessment (18 mJ/spot, 361 spots/cm^2) with one area left untreated as a control [Supplementary Figure 1, http://links.lww.com/CM9/A30]. Skin specimens were serially obtained by excision biopsy from the dorsal aspect of each mouse. Photographs were taken every other day (d1, d3, d5, d7, d9, d11, and d13) were used to evaluate hair growth. Skin histology (hematoxylin-eosin [HE] staining), immunohistochemistry, and real-time polymerase chain reaction (PCR) assays were used to determine hair follicle status and relevant molecule expression. Development of local erythema, escharosis, ulcers, or subsequent scarring were also recorded. The number of hair follicles was calculated from a fixed area (0.06 mm^2) using HE images of longitudinal sections. The anagen follicle (A) ratio was evaluated in a fixed area (0.06 mm^2) from horizontal sections. Hair follicle numbers and anagenic follicle ratios were calculated using Image J software (National Institutes of Health, USA). Expression of several inflammatory cytokines related to wound healing and Wnt signal pathway molecules was assessed by real-time PCR on the ABI 7500 qPCR system using the listed primer pairs [Supplementary Table 1, http://links.lww.com/CM9/A30]. Raw quantifications were normalized to glyceraldehyde-3-phosphate dehydrogenase values for each sample and fold changes were shown as the mean ± standard deviation (SD) of three independent experiments, each conducted in triplicate.

For immunohistochemistry tests, primary antibodies were incubated at the following dilutions: Wnt10b (1:200, Abcam ab70816; Abcam, Cambridge, UK) and vascular

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10.1097/CM9.0000000000000220

endothelial growth factor (VEGF; 1:100; Boster Biological Technology Co. Ltd., CA, USA). After treatment with a secondary antibody, sections were visualized using a DAB kit (EnVision TM Detection System; Dako Denmark A/S, Denmark), and observed under light microscope. All statistical analyses were performed with SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Results were reported as mean ± SD. Statistical significance was determined using a one-way analysis of variance. A P value <0.05 was considered statistically significant.

We next analyzed the four-beam energy doses described above for anagen promotion, skin reactions, and side effects. Results are summarized in Supplementary Figure 1, http://links.lww.com/CM9/A30. After irradiation with each beam energy setting, yellow escharosis was observed at the levels of 18 and 24 mJ/spot, respectively. The most effective dosage was 18 mJ/spot and 361 spots/cm^2. This was the setting under which premature anagen entry with gray/black color of the dorsal skin occurred on day seven and clearly visible hair re-growth occurred on day 11. In contrast, the higher energy of 24 mJ/spot induced superficial ulcers which ultimately developed into scarring. Under lower beam energies of 6 and 12 mJ/spot, only a little crusta was apparent on the dorsal skin. However, premature anagen entry could be observed from the gray/black color of the dorsal skin on day 11.

For the exploration of telogen-to-anagen conversion after fractional laser treatment, settings of 18 mJ/spot and 361 spots/cm^2 were chosen as the ideal parameter. On day 1 of laser irradiation many inflammatory cells, particularly neutrophils, immediately aggregated around microscopic thermal injury zones (MTZs) and hair follicles. On day 3, epidermis exfoliation and re-epithelialization were observed. Meanwhile, mild inflammatory infiltration into the dermis, particularly around hair follicles, was observed and progressively resolved afterward. Sequential increases in hair re-growth were observed from days 5 to 13 [Figure 1A and 1B]. Quantitatively, we found that the number of hair follicles and hair follicle anagen proportion progressively increased from day 5 backward [Figure 1C and 1D]. At the molecular level, mRNA expression of interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β1 (TGF-β1) increased immediately following fractional laser treatment and then gradually decreased. Interestingly, expression of VEGF, TGF-β1, and Wnt10b increased sharply after laser treatment, then declined and finally appeared to gradual increase from days 7 to 13 [Supplementary Figure 2, http://links.lww.com/CM9/A30]. At the protein level, increased expression in the dermis environment of VEGF and Wnt10b around hair follicles and hair follicle epithelial cells was observed on immunohistochemical analyses following fractional laser treatments [Figure 2A and 2B]. Expression of IL-1β, IL-6, and Wnt5a was negative (data not shown).

There is evidence of associations between wound healing and hair re-growth. Injury induced by the fractional laser could produce both hair follicle re-growth and scar formation in a dose-dependent manner. Moderate wounds may facilitate follicle differentiation, hair shaft production, and progression through all stages of the hair follicle cycle.[5]

Whereas severe wounds hinder hair re-growth, potentially due to the destruction of the hair follicle structure and excessive scar formation.[5] In our study, 18 mJ/spot induced hair re-growth while 24 mJ/spot developed scar formation under the same beam density [Supplementary Figure 1, http://links.lww.com/CM9/A30]. This is consistent with the conclusion of Wu et al.[5] The timing of premature anagen entry induced by the ablative CO$_2$ fractional laser (5–7 days) was earlier than that by the non-ablative 1550 nm fractional laser (7–9 days) [Supplementary Figure 1, http://links.lww.com/CM9/A30].[5] Wound healing involves three overlapping phases: inflammation, tissue formation, and tissue remodeling. Inflammation occurs immediately after injury. Various leukocyte lineages, cytokines, and growth factors are detected following to laser irradiation. Accelerated healing is accompanied with changes in epithelial tissue, inflammation, and hair follicle anagen entry.[6] It has been shown that hair follicle stem cell (HFSC) activity is regulated by both factors intrinsic to the hair follicles and extrinsic factors such as the inflammatory microenvironment.[7,8] Our results also indicate that the microenvironment of acute moderate inflammation may activate HFSC and contribute to hair regeneration. In our study, inflammatory cell aggregation around the hair follicles and MTZs, exuviate of MTZs, and re-epithelialization occurred continuously from days 1 to 3 following laser irradiation. Then, the number of inflammatory cells gradually reduced while new hair follicles regenerated and anagenic follicles ratios increased from days 5 to 11 [Figure 1A and 1B]. This indicates that anagen entry started at day 5. Similar HFSC activation induced by moderate inflammation following non-ablative fractional laser treatment was observed in the previous study.[5] Here, we speculate that moderate inflammation induced by the ablative fractional laser also activated HFSC and triggered the telogen-to-anagen conversion and hair re-growth.

In addition to the inflammation microenvironment, VEGF-mediated angiogenesis can promote hair growth and increases hair follicle size.[9] VEGF is a marker of angiogenesis, stimulating hair growth by facilitating the supply of nutrients to the hair follicle. Our data show that the expression of VEGF distributed in the dermis and hair bulb increased after laser irradiation, while the expression of IL-1β and IL-6 was negative on immunohistochemical examination [Figure 2A and 2B]. The increasing mRNA expression of VEGF and TGF-β1 after laser irradiation was divided into three stages: on day 1, on day 5, and from days 7 to 13 [Supplementary Figure 2, http://links.lww.com/CM9/A30]. This phenomenon is likely related to the anagen induction on day 5. The morphology of an anagen hair bulb started to take shape, which marked the anagen entry [Figure 1A and 1B]. Together with anagen initiation, HFSC in the bulge activated, proliferated and amplified, and then differentiated to form the new hair shaft during the anagen phase. VEGF-mediated angiogenesis and TGF-β1 signal pathway also play important roles in hair regeneration.

The Wnt/β-catenin pathway is one of the most important signaling pathways for hair growth. In a previous study, adenovirus-mediated Wnt10b over-expression-induced hair follicle regeneration. Conversely, anagen onset was
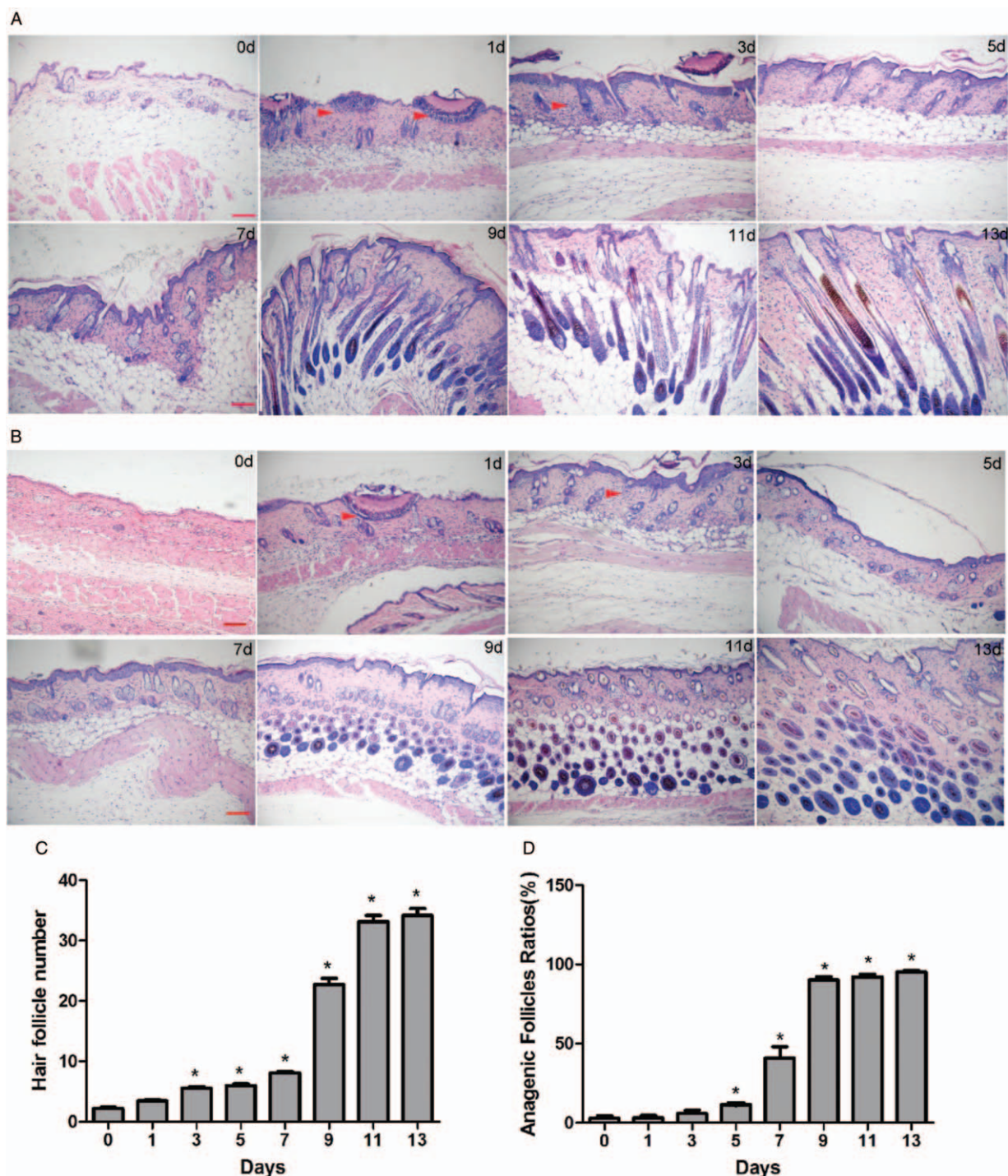
Figure 1: Histologic changes during hair cycle progression analyzed by hematoxylin-eosin staining. Beam energy: 18 mJ/spot; 361 spots/cm^2. Original magnification × 100. (A) Longitudinal sections of dorsal skin. (B) Transverse sections of dorsal skin. (C) Hair follicle numbers in deep subcutis. (D) Proportion of anagen entry ratios (%). Red arrows indicate inflammatory cells aggregation around the hair follicles and microscopic thermal injury zones, the exuviae of microscopic thermal injury zones and re-epithelialization occurred continuously from day 1 to day 3 after laser irradiation. Data shown represent mean ± standard deviation. $^*P < 0.05$ compared with day 0 ($n = 6$).

abrogated by the knockdown of Wnt10b by small interfering RNA. The Wnt10b aberrant expression data suggest this is one activator of hair follicle regeneration.[10] In this study, expression of Wnt10b increased from days 5 to 13 [Figure 2B], while expression of Wnt5a was negative (data not shown). Anagen hair bulbs were clearly visible on day 5. In terms of HFSC activation, Wnt/β-catenin signal was not sufficient on their own. It is possible that other

signals, including fibroblast growth factor, VEGF, and TGF-β coordinate with β-catenin to support HFSCs in overcoming the activation threshold.[7] Although the exact molecular mechanisms responsible for CO$_2$ factional laser-induced anagen entry remain unclear, we speculate that the inflammatory microenvironment, VEGF-mediated angiogenesis, and the Wnt10b signal pathway in combination contribute to hair re-growth. Our results suggest that
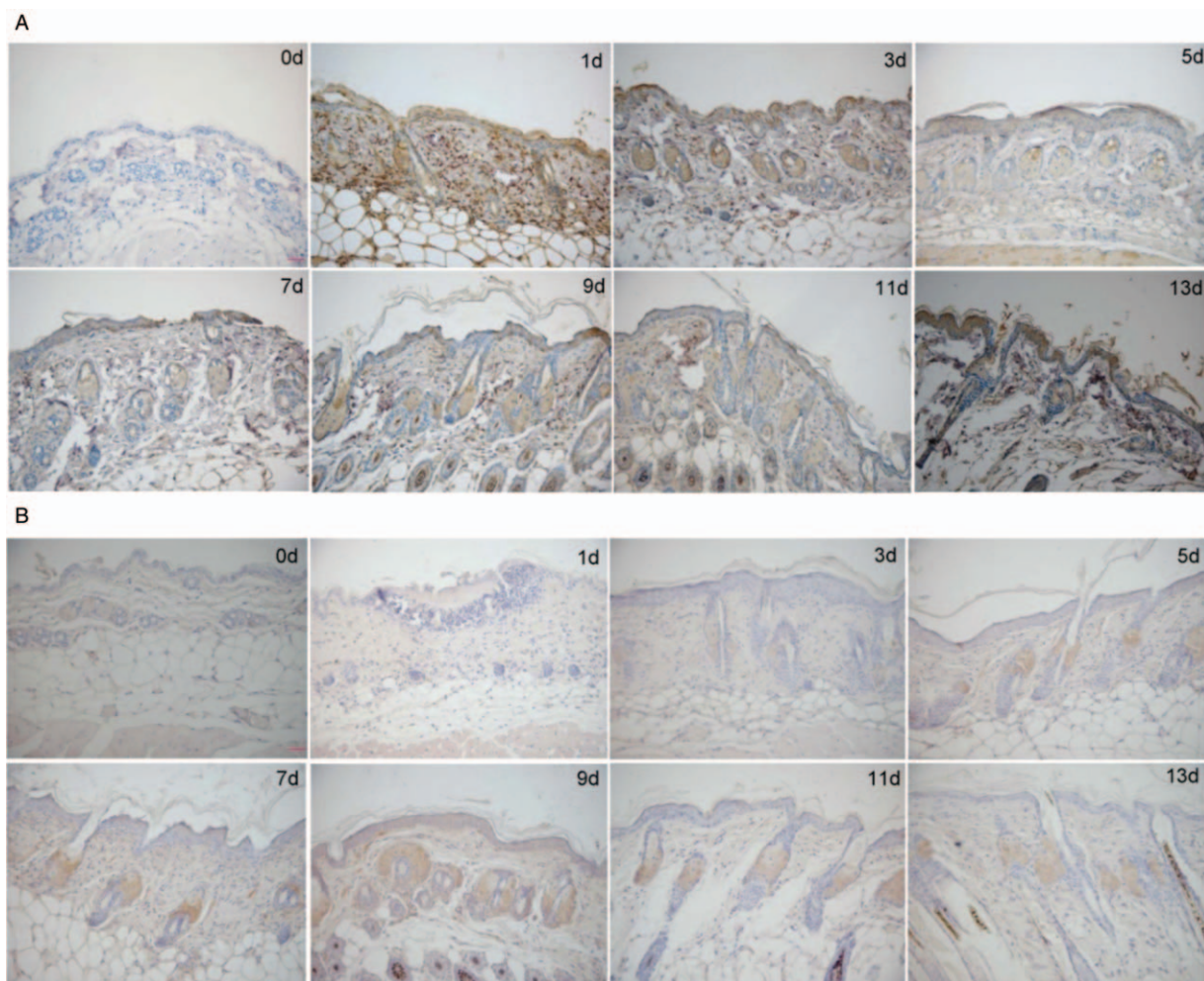
Figure 2: Expressions of vascular endothelial growth factor (A) and Wnt10b (B) shown by immunohistochemical analysis after fractional laser treatment in the dermis and hair follicles. Original magnification × 200.

ablative CO_2 fractional laser therapy may be a supplementary or alternative treatment for hair loss.

Conflicts of interest

None.

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