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**Basic Study**

# Effect of plum-blossom needle tapping with different stimulation intensities on hair regrowth in hair removal mice

## 皮肤针不同刺激量对脱毛小鼠毛发生长的影响

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**Abstract**
**Objective**: To determine the role of intensity of plum-blossom needle tapping in treating alopecia areata.
**Methods**: The BALB/c mice were randomized into a normal group, a control group, a roller-needle (RN) group, a mild plum-blossom needle (MP) group, and a heavy plum-blossom needle (HP) group. An area of hair was removed by external application of 8% sodium sulfide on BALB/c mice. The hair regrowth, hair follicle changes, and local inflammatory factor changes after cutaneous acupuncture were observed.
**Results**: After treated with sodium sulfide, the hair was completely removed, the local hair follicles reached the catagen phase, and the expressions of interleukin (IL)-1α, IL-1β, IL-2, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-17 were increased. Mice intervened by RN achieved the same hair growth rating as the controls but with thicker hair shafts; mice in the MP group had incomplete and uneven hair growth but thicker hair shafts; mice in the HP group didn't show hair growth. Pathological analysis revealed significant inflammatory infiltration into the local follicle bulbs and increased catagen-phase follicles in the control group, while RN and MP groups showed significantly increased anagen-phase follicles, coarser individual hairs, and obvious hair shafts. Meanwhile, most of the hair follicles in the HP group were in telogen phase and showed obvious surrounding inflammatory infiltration. RN, MP, and HP significantly down-regulated the increased IL-2, IL-1α, IL-1β, TNF-α, and IFN-γ levels ($P$<0.05), but didn't notably affect the increased CD34 expression ($P$>0.05).
**Conclusion:** Cutaneous acupuncture with heavy stimulation intensity can inhibit hair growth in hair removal mice, while RN, with the lightest stimulation intensity, is unlikely to affect hair growth but may make hair shafts thicker and follicles larger.
**Keywords**: Alopecia; Cutaneous Acupuncture; Plum-blossom Needle Therapy; Roller Needle; Interleukin; Inflammation; Mice

**【摘要】目的：**不同针刺刺激量的皮肤针治疗对脱毛小鼠毛发生长的影响。**方法：**BALB/c 小鼠随机分为正常组、模型对照组、滚针组、梅花针轻叩组、梅花针重叩组。8%硫化钠外涂使小鼠局部脱毛。皮肤针治疗后观察毛发生长、毛囊改变及局部炎症因子变化。**结果：**小鼠局部经 8%的硫化钠涂抹后毛发完全脱落，毛囊进入退行期，炎症细胞因子 IL-1α、IL-1β、IL-2、TNF-α、IFN-γ、IL-17 表达增加。滚针组小鼠毛发生长完全程度与模型对照组相同，但毛干较模型对照组更粗大；梅花针轻叩组小鼠毛发生长不完全、欠均匀，但毛干较模型对照组粗大；梅花针重叩组小鼠毛发未生长。病理显示对照组脱毛小鼠局部毛囊球部炎症浸润明显，进入退行期的毛囊增多；滚针组、梅花针轻叩组与对照组比较，处于生长期的毛囊明显增多、个体粗大，并可见明显毛干；梅花针重叩组毛囊多进入静止期，且周围炎性浸润明显。免疫组化显示滚针、梅花针轻叩、梅花针重叩治疗均能使增高的 IL-2、IL-1α、IL-1β、TNF-α、IFN-γ 下降($P$＜0.05)，但对增高的 CD34 表达无明显作用($P$＞0.05)。**结论：**刺激量大的梅花针重叩抑制了毛发的生长；刺激量最轻的滚针，不影响毛发生长，而且能使毛干、毛囊更粗大。
**【关键词】**秃发；皮肤针疗法；梅花针疗法；滚针；白细胞介素；炎症；小鼠
**【中图分类号】**R2-03　　**【文献标志码】**A

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Plum-blossom needle tapping is an external treatment with a long history in traditional Chinese medicine by tapping the body surface and meridians using a plum-blossom needle to treat diseases including alopecia areata.

Clinical studies have reported that plum-blossom needle acupuncture or/and drugs are useful for alopecia areata and other decalvant disorders[1-4]. But no literatures have indicated which stimulation intensity is more suitable when applying plum-blossom needle tapping. So, effects of different stimulation intensities are worthy of study.

Thus, here we applied a depilatory to locally removed hair of mice and observed the effect of stimulation intensity on hair regrowth as well as the changes of inflammatory factors surrounding the hair follicles in the hair removal area.

## 1 Materials and Methods

### 1.1 Animals

A total of 50 male SPF BALB/c mice (6-8 weeks old, mean body weight 19.5 g) were obtained from Shanghai Laboratory Animal Center. These mice had no obvious genetic or systemic diseases. Each mouse appeared well prior to the experiment and was raised in the Laboratory Animal Center, the Third People's Hospital Affiliated to Shanghai Jiao Tong University. The mice were randomly divided into a normal group, a control group, a roller-needle acupuncture (RN) group, a mild plum-blossom needle (MP) group, and a heavy plum-blossom needle (HP) group, 10 mice in each group.

### 1.2 Reagents

Sodium sulfide (Shanghai Zhanyun Chemical Co., Ltd., China); paraffin wax, formaldehyde, dimethylbenzene, 30% $H_2O_2$, absolute ethanol and ammonium hydroxide (Sinopharm Chemical Reagent Co., Ltd., China); hematoxylin-eosin (HE) (BASO Company, China); neutral resin, broad-spectrum second antibody, and enhanced 3,3′-diaminobenzidine (DAB) chromogenic kit (Shanghai Long Island Biotech. Co., Ltd., China); anti-tumor necrosis factor (TNF)-α, interferon (IFN)-β, interleukin (IL)-α, IL-β, and IL-2 primary antibodies as well as rabbit anti-mouse second antibody (Santa Cruz Biotechnology, CA).

### 1.3 Modeling method

Hair of 2 $cm^2$ at the junction of the right hip was removed after applied with 8% sodium sulfide[5-6]. Dry cotton wipes were used to scrub the treated area 3-4 times to remove the hair and the residual sodium sulfide solution was also removed (Figure 1).

### 1.4 Interventions

#### 1.4.1 Plum-blossom needle treatment

The plum-blossom needle treatment started on the 3rd day after modeling. Each mouse was held in a researcher's left hand and placed on the cover of a chicken-wire cage with the tail held for stability. A 75% alcohol cotton ball was held in the researcher's right hand and used to disinfect the hair removal site. A plum-blossom needle was then used to tap on the site. The tapping force was relatively lighter (to cause slight visible bleeding) in the MP group and relatively heavier (to cause significant petechial bleeding) in the HP group. The treatment lasted 20-30 s each time and was administered three times per week for a total of 3 weeks.

#### 1.4.2 Roller needle treatment

The roller needle treatment started on the 3rd day after modeling. Each mouse was held in a researcher's left hand and placed on the cover of a chicken-wire cage with the tail held for stability. A 75% alcohol cotton ball was held in the researcher's right hand and used to disinfect the hair removal site. The roller needle was applied mildly across the site with the mouse held firmly to prevent pain-induced struggles. The treatment lasted 20-30 s each time and was administered three times per week for a total of 3 weeks.

### 1.5 Observation items

#### 1.5.1 Histological observation

Hair growth was observed and photographed daily. The mice were executed by cervical dislocation and the skin at the hair removal site was obtained and fixed in 10% formalin, followed by paraffin-embedding, slicing, and HE staining.

#### 1.5.2 Immunohistochemical (IHC) analyses

The samples were collected, fixed, sliced, HE stained, dewaxed with dimethylbenzene, and sealed with 3% $H_2O_2$ for 5 min. The antigens were retrieved in 95 °C water bath using ethylenediamine tetraacetic acid (pH =9.0) for 40 min, followed by addition of the primary antibody at 4 °C overnight, addition of the secondary antibody for 30 min, DAB rendering, and HE counterstaining. For the negative control, phosphate buffered saline replaced the primary antibody. Positive results would be determined if brown particles were seen in the membrane or cytoplasm.

### 1.6 Statistical analysis

Statistical analyses were performed using SPSS 20.0 software. Measurement data were performed by using completely random control analysis of variance. Data were subjected to a homogeneity test for variance, and those with heterogeneity of variance were subjected to rank-sum test. $P < 0.05$ indicated a statistical significance.

## 2 Results

### 2.1 Local hair removal

After the 8% sodium sulfide was applied and left to stand for 2 min, the site was cleaned with a dry cotton

ball to expose the pink skin. There were no visible defects, redness, swelling, or corrosion. Forty-eight hours later, no obvious abnormalities were visible. (Figure 1).

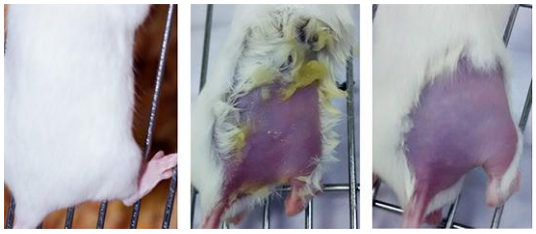


**Figure 1. Hair removal by 8% sodium sulfide and skin response in BALB/c mice**

HE staining revealed that, compared with those in the normal group, mice in the control group showed hair loss and catagen-phase follicles, but the difference between 0 h and 48 h was not statistically significant (Figure 2).

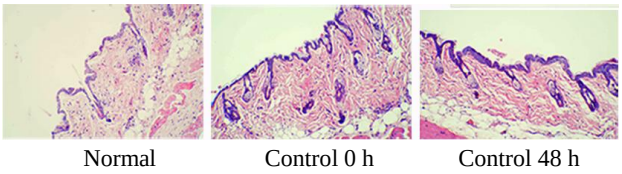


**Figure 2. HE staining histology and local inflammation after hair removal in BALB/c mice (HE, ×100)**

IHC analysis demonstrated that, the expressions of inflammatory cytokines IL-1α, IL-1β, IL-2, TNF-α, IFN-γ, and IL-17 increased in the control group compared with those in the normal group after treated by 8% sodium sulfide. However, the difference did not take place between 0 h and 48 h (Figure 3 and Figure 4).

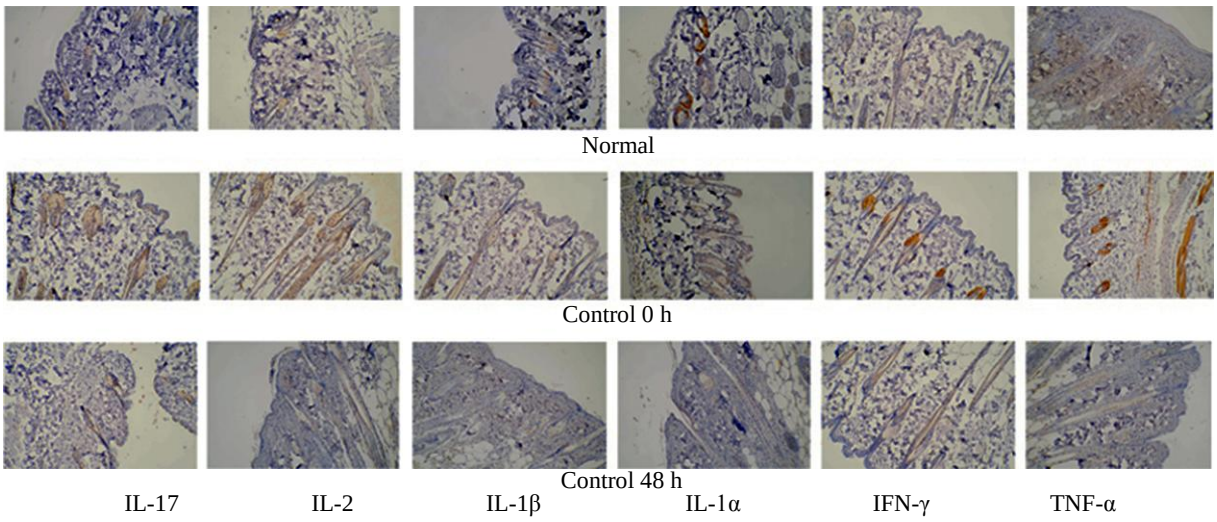


**Figure 3. IHC staining histology and local inflammation after hair removal in BALB/c mice (IHC, ×100)**

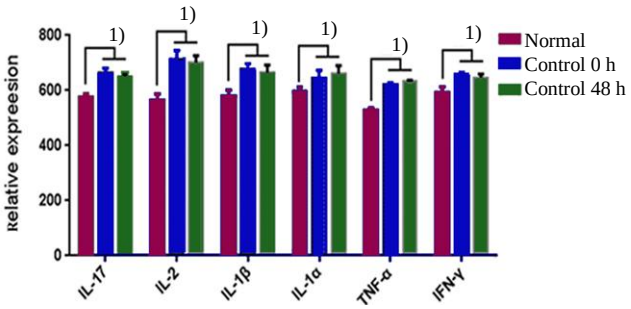


**Figure 4. Relative expression**

Note: Compared with the normal group, $P<0.01$

## 2.2 Manifestation after different interventions

Cutaneous acupuncture might cause slight damage to skin in human as we have known, and similar phenomenon was also observed in our study. After the intervention, mice in the RN group demonstrated a tiny amount of bleeding on the skin, a small number of bleeding points were seen in the MP group, and a large number of bleeding points on the skin, obvious subcutaneous bleeding and bruises, and edema at the tapping site were observed in the HP group (Figure 5).

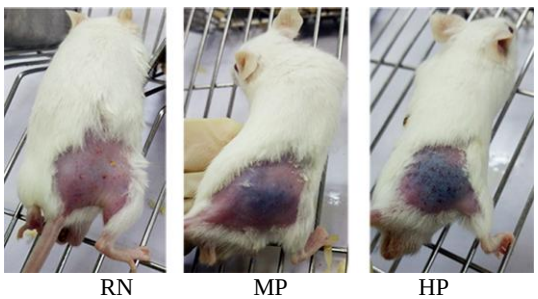


**Figure 5. Skin response after receiving different stimulation intensities of plum-blossom needle tapping**

## 2.3 Hair regrowth

Five to seven days after modeling, hair gradually

 

grew in each group. Twenty-one days after modeling, the hair growth was substantial. The new hair was uniform and dense in the control group, which was natural. The hair growth in the RN group was at the same level compared with that in the control group but with thicker hair shafts. The MP group had incomplete and uneven hair growth but thicker hair shafts, while the hair regrowth had been completely inhibited by heavy plum-blossom needle treatment as no hair growth could be seen in the HP group (Figure 6).

HE staining revealed that, compared with the normal group, mice in the control group demonstrated significant inflammatory infiltration at local follicle bulbs and increased numbers of catagen-phase follicles. Compared with the control group, the RN and MP groups showed significantly increased anagen-phase follicles, coarser hairs, and obvious hair shafts. Meanwhile, most of the hair follicles in the HP group were in telogen phase and demonstrated obvious surrounding inflammatory infiltration (Figure 7).

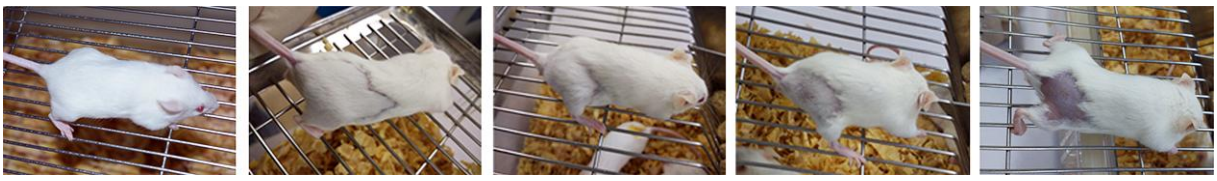


**Figure 6. Effects of different plum-blossom needle tapping on hair regrowth**

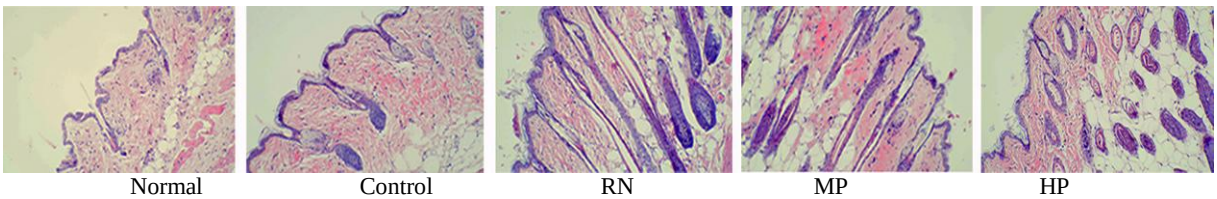


**Figure 7. Histology and local inflammation after different plum-blossom needle tapping (HE, ×100)**

### 2.4 Changes in local inflammation

IHC detection revealed that after sodium sulfide were applied to remove the hair, the expressions of the inflammatory cytokines IL-1α, IL-1β, IL-2, TNF-α, IFN-γ, and IL-17 increased in the control group, which suggested an obvious inflammatory response. On the other hand, the expressions of IL-2, IL-1α, IL-1β, TNF-α, and IFN-γ were significantly decreased after roller needle treatment, mild plum-blossom needle treatment, and heavy plum-blossom needle treatment ($P<0.05$), indicating alleviated inflammation. Although the IL-17 expression was not significantly decreased after heavy plum-blossom needle treatment ($P>0.05$), the IFN-γ expression was alleviated to a normal level (Figure 8 and Figure 9) .

### 2.5 Effects of plum-blossom needle tapping on CD34 expression

The expression of CD34, a specific marker of hair follicle stem cells in mice, was evaluated. IHC study demonstrated that, compared with that in the normal group, CD34 expression increased after modeling ($P<0.01$), whereas no significant difference showed among the control, RN, MP, and HP groups after the interventions in comparing the CD34 expression (all $P>0.05$), indicating that plum-blossom needle tapping produced no significant influence on it (Figure 10 and Figure 11).

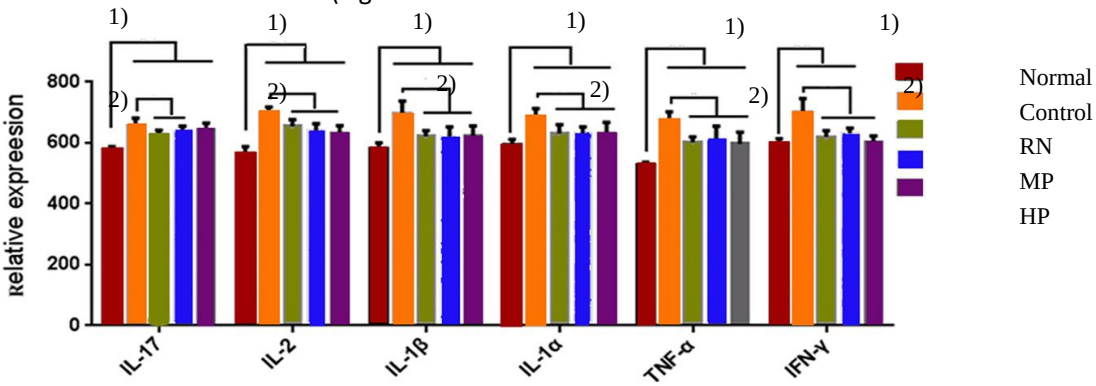


**Figure 9. Comparison of expressions of the inflammatory cytokines after different cutaneous acupuncture therapies**

Note: Compared with the normal group, 1) $P<0.01$, 2) $P<0.05$

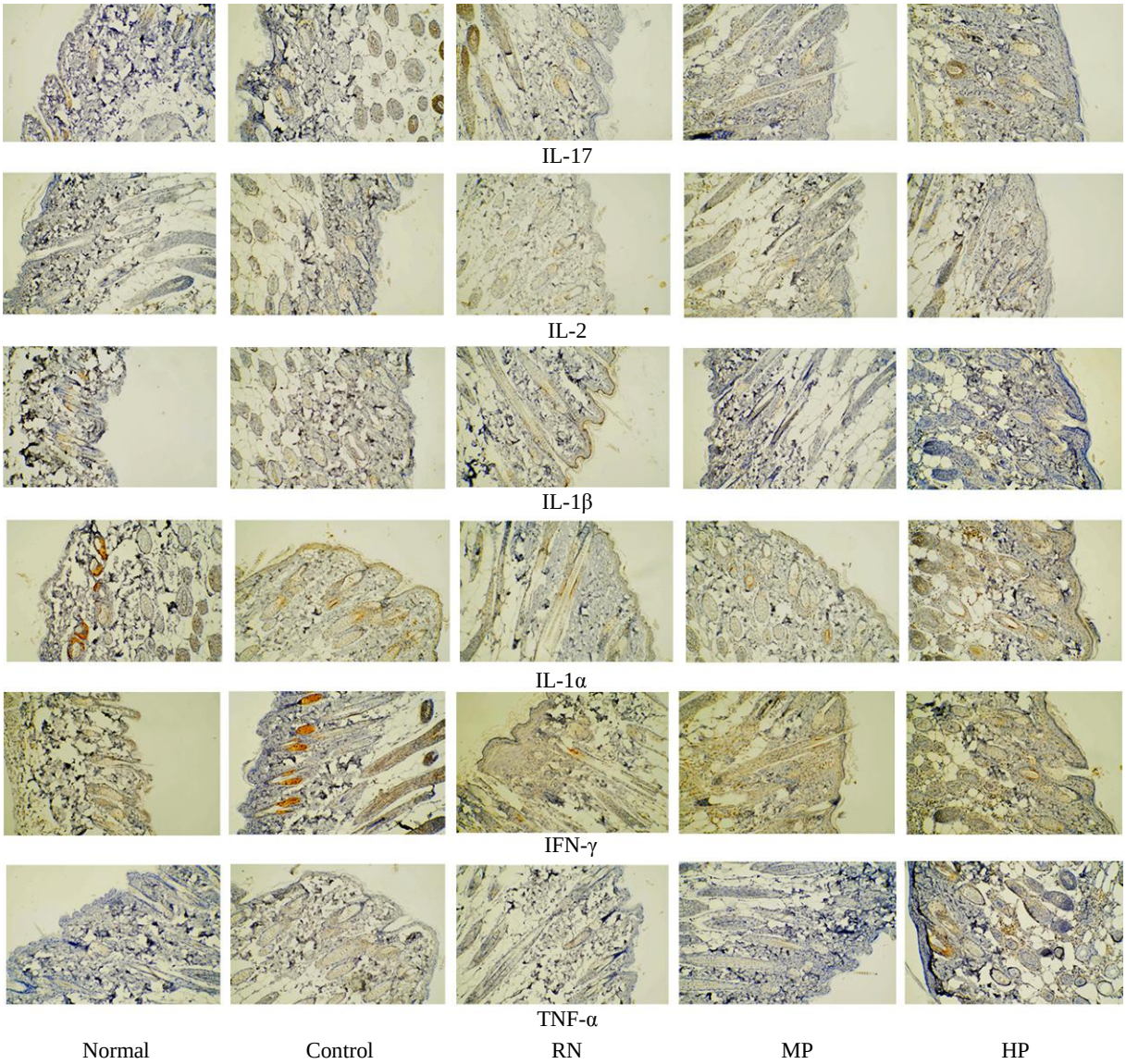


**Figure 8. Expressions of inflammatory cytokines after different cutaneous acupuncture therapies (IHC, ×100))**

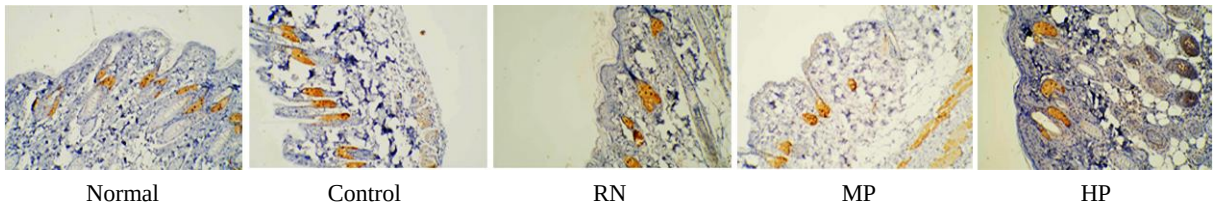


**Figure 10. CD34 expression after different cutaneous acupuncture therapies (IHC, ×100)**

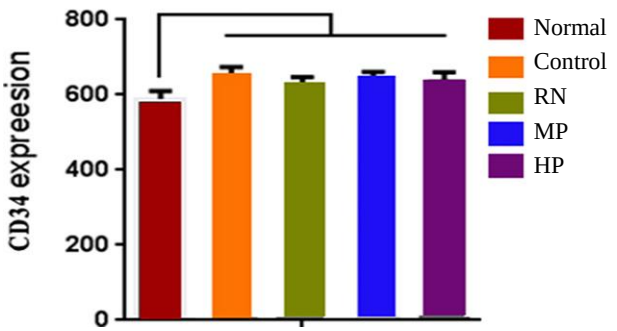


**Figure 11. Comparison of CD34 expression after different cutaneous acupuncture therapies**

Note: Compared with the normal group, 1) $P<0.01$

## 3 Discussion

Dermal needling therapy refers to a type of acupuncture treatment by tapping the skin surface with special needles, including plum-blossom needle, roller needle and intradermal needle, to treat diseases.

 

Plum-blossom needle is a type of dermal needling needle with five or seven needle tips on the top. Roller needle is a modified plum-blossom needle with numerous tiny needles fixed on a small roller to roll across the body's surface for treatment. The difference between them is the stimulation intensity: roller needle can achieve a milder stimulation than that plum-blossom needle. Different from plum-blossom needle and roller needle in the way of action, intradermal needle therapy is performed by embedding a small needle subcutaneously for one or several days. We can achieve mild and heavy stimulation intensities by adopting plum-blossom needle, but lower stimulation intensity was still needed. Thus, the RN group was designed.

Some studies developed spontaneous alopecia areata model by using elderly inbred C3H/HeJ mice, but the yield was very low[7-8]. The existing studies on the mechanism of acupuncture treatment for alopecia areata didn't provide modeling approach[9]. Therefore, in this study, we developed the alopecia areata model by external application of 8% sodium sulfide[6-7], in order to observe the effect of cutaneous acupuncture on hair regrowth.

Alopecia areata is a non-scarring and inflammatory decalvant disease[10] with a very complex mechanism. However, there are two main pathological processes in patients with alopecia areata: immune reaction and hair follicle degradation. The proportions of catagen-phase and telogen-phase hair follicles are increased in alopecia areata. In the catagen phase, hair matrix disappears and hair papillae swell; in the chronic stage, hair follicles appear malnourished and the morphological changes of hair shaft and vellus hair follicles become visible. The current two-step inflammation theory states that the inflammation surrounding hair follicles is the basic pathological process of alopecia areata[11].

Studies to date on alopecia areata associate cytokines mainly focused on IL-1, IL-2, TNF-α, IFN-γ[12-14], and IL-17[15-18], which are highly expressed in human and experimental animals. The levels of these cytokines basically reflect the immunopathological process of alopecia areata. Previous experimental study found that electroacupuncture at Zusanli (ST 36) point of a mouse model of spontaneous alopecia areata can improve the inflammatory responses in follicles in hair removal area and its mechanism might be associated with the reduced proliferation of mast cells[9].

In this experiment, we compared the effects of plum-blossom needle treatment with different stimulation intensities versus roller needle treatment on hair regrowth and local inflammation. The results showed that the hair regrowth in the RN group was basically consistent with that in the control group, but the hair was thicker, while the hair growth was totally inhibited in the HP group. The HE staining results were consistent with the observed phenomenon of hair growth. IHC results showed that, roller acupuncture, mild plum-blossom needle treatment and heavy plum-blossom needle treatment significantly lowered the increased inflammatory cytokines levels including IL-2, IL-1α, IL-1β, TNF-α, and IFN-γ (all $P<0.05$), suggesting that the inflammation can be reduced by plum-blossom needle tapping.

These findings revealed that the effectiveness of roller needle acupuncture with the lightest stimulation intensity was better than that of heavy plum-blossom needle treatment, meaning that high-intensity plum-blossom needle treatment is likely to inhibit hair regrowth. It was indicated that cutaneous acupuncture of heavy stimulation should not be applied for alopecia.

We had been very concerned about whether cutaneous acupuncture has influence on hair follicle stem cells, the seed cells for hair follicle growth and development at the beginning of the study. Theoretically, the abnormalities of hair follicle stem cells affect hair formation and development and are associated with decalvant diseases. Studies have revealed that CD34 is a specific marker of hair follicle stem cells in mice[19-21]. However, our study found that the effect of roller needle acupuncture, mild plum-blossom needle treatment, and heavy plum-blossom needle treatment on CD34 expression was not significant.

In addition, this experiment was based on an acute hair removal model, and the mice have self-healing abilities. Thus, there expects a model with long-term hair growth inhibition to study the role of roller and plum-blossom needles.

For further research, the effects of acupuncture on other pathways that reflect hair growth are still required to investigate. For example, it's reported that the inhibition of JAK-STAT pathway would regulate the activation of hair follicle stem cells and promote hair growth[22]; hair growth could be promoted by inducing hair cycle transition from telogen to anagen phase in C57BL/6 mice through the regulation of Wnt 10b and β-catenin[23]. Whether the mechanism of cutaneous acupuncture in treating alopecia is closely related to the pathways mentioned above needs further studies.

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**Conflict of Interest**

There was no potential conflict of interest in this article.

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**Statement of Human and Animal Rights**

The study was conceived according to the principles of the ‘three Rs’ (replacement, reduction and refinement) and all animal procedures were performed in accordance with the ‘Guide for the Care and Use of Laboratory Animals’.

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