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탈모마우스모델에서의 송지추출물 및 그 성분인 아비에트산의 모발성장효과

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Hair Growth-promoting Effect of Resina Pini and Its Main Constituent, Abietic Acid, in Mouse Model of Alopecia

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요 약: 최근 남성성탈모증에 대한 관심이 증대되고 있으며, 이에 천연물 및 그의 활성성분을 활용한 새로운 약물 개발에 대한 연구가 증가하고 있다. 송지(Resina Pini, RP)는 *Pinus* sp. (Pinaceae)의 수지질로 전통의학적으로 감염, 우식증, 치주질환에 사용되어왔다. 본 연구진은 RP의 성분인 아비에트산(abietic acid, AA)이 남성성탈모기전에 중요한 효소인 5α-reductase를 억제하는 효과를 세포 수준에서 입증한바 있으며, 이번 연구에서는 실제로 탈모억제 및 모발 성장에 대하여 실험동물 수준에서 입증하고자 한다. C3H/HeN 탈모마우스 모델에서 RP는 300 mg/kg에서 유의하게 탈모억제를 확인하였으며, 뿐만 아니라 AA는 30 mg/kg에서도 유의하게 탈모억제효과를 보였다. 이상의 결과로부터 RP는 그 활성성분인 AA가 5α-reductase 억제하는 기전을 통해 남성성탈모억제효과를 보였다고 사료되며, 향후 탈모억제 보완치료법으로의 이용 가능성을 보였다.

Abstract: Recently, increased attention has been directed toward medicinal extracts and their active ingredients as potential new drug candidates for androgenic alopecia. Resina Pini (RP), a resinous exudation obtained from *Pinus* sp. (Pinaceae), has been used as a traditional medicine for the treatment of infection, pain related to dental caries, and periodontal disease. Previously, we suggested that RP and its main constituent, abietic acid (abieta-7,13-dien-18-oic acid; AA), may play important roles against androgenic alopecia as 5α-reductase inhibitors. However, to date, there is no evidence that AA has hair growth-promoting effects *in vivo*. In this study, we found that 10 ~ 300 mg/kg RP and 3 ~ 30 mg/kg AA significantly promoted hair growth in a C3H/HeN mouse model of alopecia. To our knowledge, this is the first report of the hair growth-promoting effects of RP and AA *in vivo*. From these results, RP and its main constituent AA can promote hair growth in mouse by inhibiting 5α-reductase activity and may be effective alternative therapies for androgenic alopecia.

Keywords: *hair growth, Resina Pini, abietic acid, androgenic alopecia, mouse model, 5α-reductase inhibitor*

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1. Introduction

Alopecia, also known as hair loss or baldness, is a dermatological disorder with a global distribution; it affects approximately 0.2 ~ 2% of the global population[1]. Alopecia has many causes including androgenic alopecia (AGA), fungal infection, trauma, radiotherapy, chemotherapy, nutritional deficiencies, and autoimmune diseases such as alopecia areata[2]. AGA is an androgen-linked condition in genetically prone individuals; it affects 50% of the male population[1,2]. A number of genetic and environmental factors play a role in AGA development. AGA is a frequent form of alopecia in which androgens progressively convert normal-sized scalp hair follicles to miniaturized hair follicles[3,4]. Dermal papilla cells are mainly affected by a specific androgen, 5α-dihydrotestosterone. It is produced from testosterone in dermal papilla cells by the catalytic action of 5α-reductase type-2 enzyme[2]. The 5α-reductase type-2 enzyme plays a vital role through the intracellular conversion of testosterone to 5α-dihydrotestosterone within the follicular epithelium[2]. Hair loss is characterized by the shortening of the anagen phase and miniaturization of hair follicles, resulting in thinner and shorter hairs. Therefore, pharmacological inhibition of 5α-reductase type-2 could, potentially, be a promising strategy for AGA prevention.

Resina Pini (RP), a resinous exudate obtained from *Pinus* sp. (Pinaceae), has traditionally been used to control infection, inflammation, and pain related to dental caries and periodontal disease. RP could be an effective alternative to conventional therapeutic agents for periodontal tissue inflammation and breakdown[5,6]. In addition, RP inhibited the PGE_2 production in human gingival fibroblasts and their analgesic effect on acetic acid-induced writhing in mice[7]. We have demonstrated previously that the 50% ethanol extracts from RP showed inhibitory activity against 5α-reductase prepared from rat prostate[6]. Further, the fraction responsible for this activity was purified, and the active constituent was isolated and identified as abietic acid (abieta-7,13-dien-18-oic acid, AA), which exhibited potent 5α-reductase inhibitory activity *in vitro*[6]. However, to date, there is no evidence that AA has a hair growth-promoting effect in a C3H/HeN mouse model of alopecia. In the present study, we investigated the effects of RP and its main constituent AA on hair growth promotion.

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin / streptomycin (P/S) and fetal bovine serum (FBS), were purchased from Gibco (MD, USA). Minoxidil, ethanol, MTT, and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Also, PEG_2 was purchased from Abcam (Cambridge, MA). The other reagents used were of guaranteed or analytical grade. The RP extract and abietic acid were the same as those used in our previous study[6].

2.2. Cell Culture

The macrophage RAW 264.7 cell line was purchased from ATCC (Manassas, VA). Cells were kept in DMEM supplemented with 10% heat inactivated FBS and 1% P/S in an atmosphere of 95% air and 5% CO_2 at 37 ℃. All experiments were conducted 12 h after the cells had been seeded on the 96- and 24-well plates at densities of 1×10^4 and 2×10^4 cells/well respectively.

2.3. Assessment of MTT, NO, and PGE_2 Assay

MTT and NO generation were generated as the previously described method[8]. Then, PEG_2 levels was determined using a PGE_2 ELISA kit, according to the instruction manual.

2.4. Animals and Treatments

Animal maintenance and treatment were carried out in accordance with the principles of laboratory animal care (NIH publication No. 85 ~ 23, revised 1985), the association for assessment and accreditation of laboratory animal care (I-0812030) system and the animal care and use

guidelines of Dong-A Pharmaceutical Co., LTD. (Seoul, Korea). Female C3H/HeN mice (9 weeks, 20 ~ 22 g) were purchased from Jung-Ang Lab. Animal Inc. (Seoul, Korea). Animals were housed at an ambient temperature of 23 ± 1 ℃ and relative humidity of 60 ± 10% under a 12 h light/dark cycle, and were allowed free access to water and food. Animals were assigned to nine groups; (1) group 1 (non-treated group), (2) group 2 (vehicle (50% ethanol) only-treated group), (3) group 3 (1% minoxidil-treated group), (4) group 4 (RP 10 mg/kg/day treated group), (5) group 5 (RP 100 mg/kg/day treated group), (6) group 6 (RP 300 mg/kg/day treated group), (7) group 7 (AA 3 mg/kg/day treated group), (8) group 8 (AA 10 mg/kg/day treated group), and (9) group 9 (AA 30 mg/kg/day treated group). After complete removal of the dorsal hairs within an area of approximately 8 cm^2, 150 μL of each solution was applied to the dorsal skin of the mice twice daily for three weeks. On days 5, 9, 12, 15, 19, and 22 after starting application, a hair growth score was given to each mouse. For the assessment of experimental variability, three independent experiments were carried out in duplicate.

2.5. Evaluation of Hair Growth Scores

The four signs of skin lesions were (1) no hair growth pink skin, (2) skin color changes from pink to gray or black without visible hair growth, indicating the onset of anagen, (3) sparse or diffuse short hair growth, and (4) dense, normal coat hair, and hair growth activity. The above-mentioned symptoms were graded as follows: 0 (no progress), 1 (mild), 2 (moderate), and 3 (fully progressed).

2.6. Statistical Analysis

The statistics were expressed as the mean ± S.D. error of the mean (S.E.M). The statistical variables were analyzed using a one-way analysis of variance (ANOVA) and post hoc multiple mean comparisons (Tukey's HSD test). The statistical significance was set at $p < 0.05$. All variables were analyzed using the GraphPad Prism 5.10 software (GraphPad Software Inc., San Diego, CA, USA).

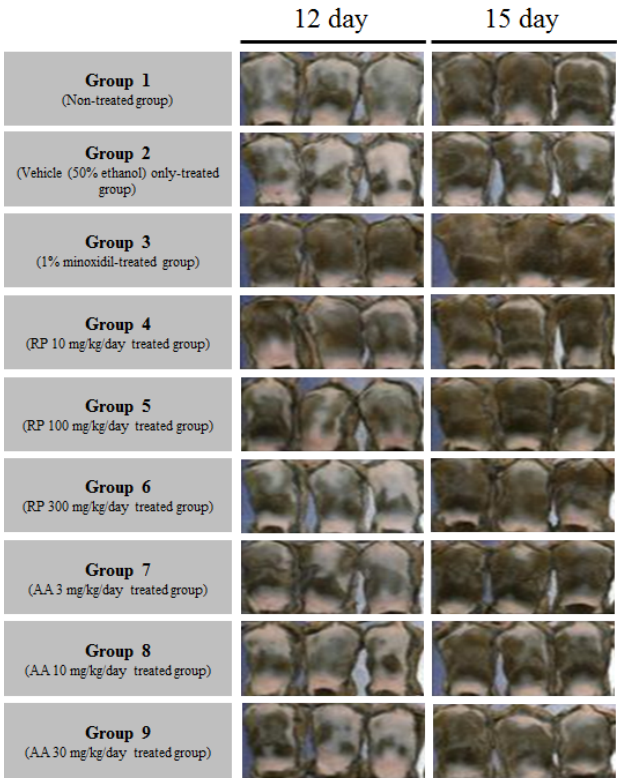

Figure 1. Macroscopic evaluation of the hair growth-promoting effects of Resina Pini and its main constituent abietic acid in C3H/HeN female mouse model of alopecia.

3. Results and Discussion

AGA is a very important aesthetic and psychological problem that often decreases the quality of life in many women[9,10]. The androgen dependent mechanism is one of the most important etiological factors of AGA, and the inhibition of 5α-reductase is, at the moment, the most efficacious way to treat AGA[9-11]. Topical minoxidil (5% solution) and oral finasteride have been approved by the food and drug administration for the treatment of AGA[1]. It has the possibility of a high affinity for androgen receptors, but as steroid derivate is expected to produce anti-androgen side effects and to be teratogenetic in fertile women[9,11]. Also, topical minoxidil, an adenosine triphosphate-sensitive potassium channel opener, was shown to be effective in 54% of treated patients as opposed to 34% in placebo control groups[1]. However,

Table 1. Hair Growth Score of C3H/HeN Female Mouse Model of Alopecia after Topical Treatment of Test Samples for 3 Weeks

	0 day	5 day	9 day	12 day	15 day	19 day	22 day
No treatment	0	0.04 ± 0.04	0.14 ± 0.12	0.79 ± 0.31	1.64 ± 0.55	2.14 ± 0.44	2.21 ± 0.45
Vehicle	0	0.11 ± 0.05	0.29 ± 0.15	0.79 ± 0.27	1.36 ± 0.38	2.21 ± 0.39	2.46 ± 0.29
1% Minoxidil	0	0.11 ± 0.08	0.71 ± 0.29*	1.96 ± 0.53	2.00 ± 0.52	2.46 ± 0.31	2.71 ± 0.23
Resina Pini 10 mg/kg	0	0.07 ± 0.05	0.36 ± 0.19	1.32 ± 0.42	1.68 ± 0.50	2.07 ± 0.46	2.64 ± 0.26
Resina Pini 100 mg/kg	0	0.04 ± 0.04	0.32 ± 0.19	1.32 ± 0.44	1.89 ± 0.46	2.46 ± 0.36	2.68 ± 0.19
Resina Pini 300 mg/kg	0	0.04 ± 0.04	0.39 ± 0.22	1.43 ± 0.36	1.93 ± 0.32	2.75 ± 0.14	2.89 ± 0.08
Abietic acid 3 mg/kg	0	0.14 ± 0.10	0.54 ± 0.27	1.29 ± 0.45	1.71 ± 0.48	2.25 ± 0.43	2.46 ± 0.35
Abietic acid 10 mg/kg	0	0.04 ± 0.04	0.21 ± 0.12	1.11 ± 0.39	1.61 ± 0.46	2.21 ± 0.48	2.50 ± 0.35
Abietic acid 30 mg/kg	0	0.07 ± 0.08	0.43 ± 0.33	1.18 ± 0.33	1.68 ± 0.41	2.36 ± 0.40	2.71 ± 0.23

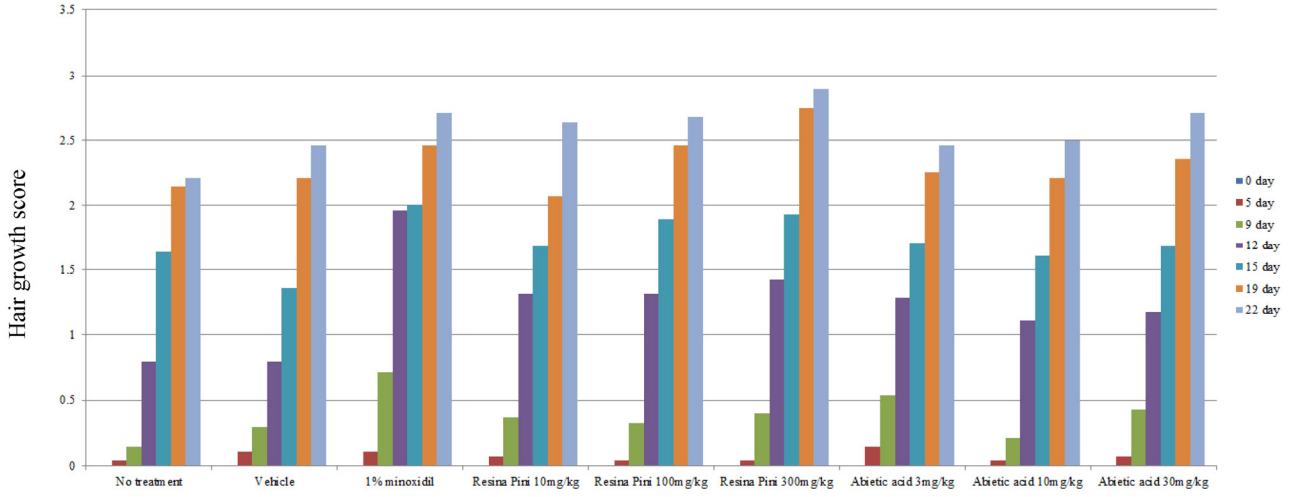

Figure 2. Hair growth score of C3H/HeN female mouse model of alopecia after topical treatment of test samples for 3 weeks. $^{*}p < 0.001$ compared with the vehicle group.

there are significant adverse dermatological effects associated with minoxidil, including pruritis, dryness, scaling, local irritation, and dermatitis. In addition, finasteride, a competitive inhibitor of type-2 5α-reductase, is known to increase hair growth in patients with androgenic alopecia[1]. It was reported that 48% of hair regrowth was observed in finasteride recipients in one year[12]. Finasteride is generally well tolerated by patients, but some patients withdrew from treatment due to drug-related sexual disorders[2]. Therefore, there remains a demand for highly effective pharmacotherapy for treating androgenic alopecia with an excellent safety and efficacy profile. In the past several years, there have been numerous attempts to develop new agents capable of preventing and/or treating androgenic alopecia[2]. For example, natural extracts from several plants have been used for hair growth promotion via inhibiting 5α-reductase activity, including *Phyllanthus niruri*, *Cacumen platycladi*, Red ginseng, and *Adiantum capillus-veneris*[13-16].

In a previous study, RP extract (50 ~ 500 μL/mL) inhibited 5α-reductase activity *in vitro* (0.8 ~ 78.2%)[6]. Furthermore, AA inhibited 5α-reductase activity *in vitro* (IC_{50} 56 μM)[6]. Although RP have inhibitory effects *in vitro* 5α-reductase assay, its potential hair growth-promoting effect has not been tested *in vivo* animal model. Therefore, in this study, we applied the hair growth-

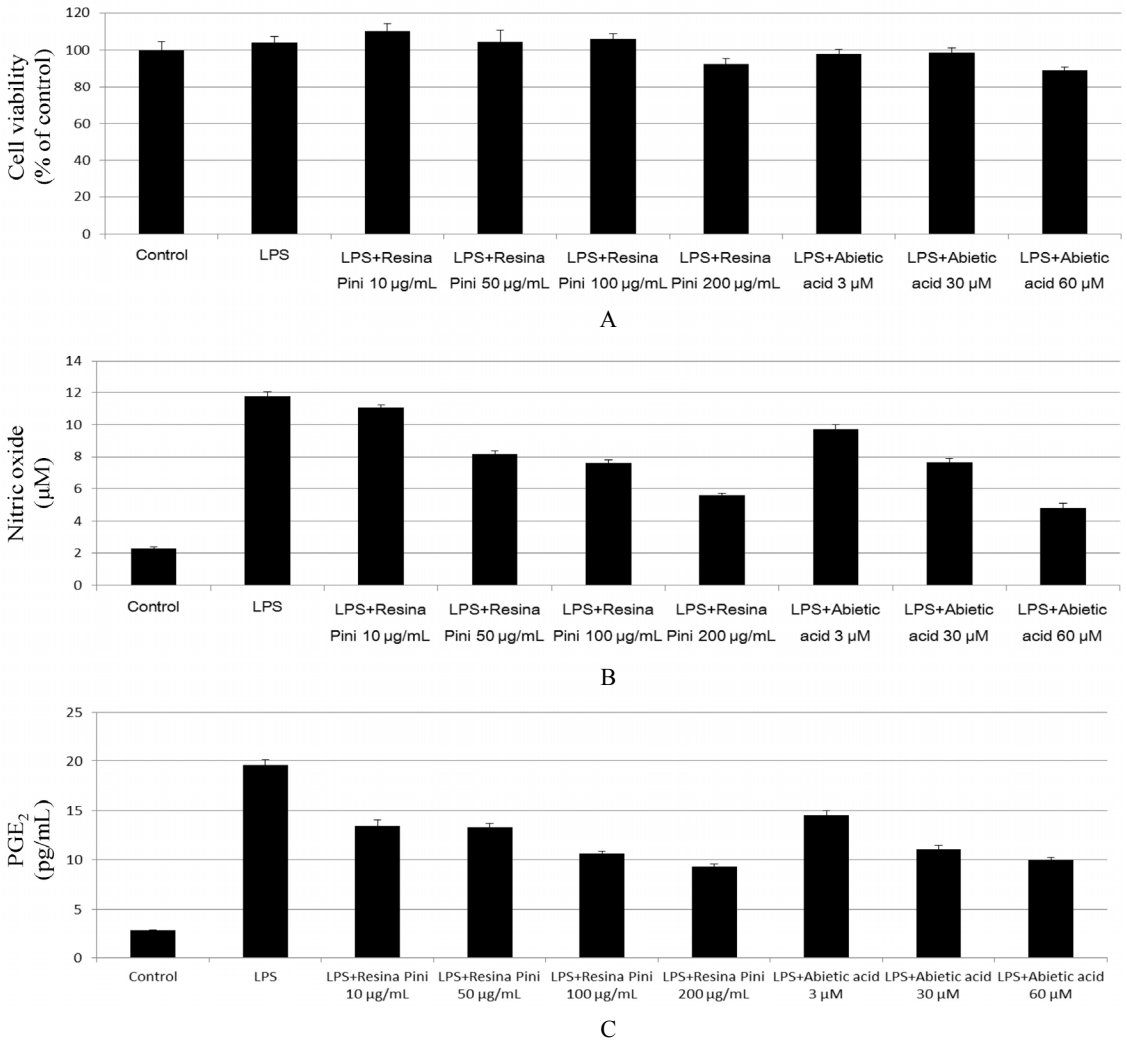

Figure 3. Inhibitory effect of Resina Pini on LPS-induced PGE_2 and nitric oxide levels in RAW 264.7 cell line. Cytotoxicity was determined based on the level of (A) MTT. (B) PGE_2 and (C) NO production was assayed. Values are presented as means ± S.E.M. $^{***}p < 0.001$ compared with the control group, $^{###}p < 0.001$, compared with the LPS-only treatment group.

promoting activity of RP and AA in alopecia models of C3H/HeN mice. The hair growth indexes of 10 ~ 300 mg/kg RP and 3 ~ 30 mg/kg AA groups were slight than that of the vehicle group after treatment for 3 weeks, indicating the hair growth-promoting activity of RP extract and AA (Figure 1, 2 and Table 1). Also, 1% minoxidil group after treatment for 3 weeks significantly promoted hair growth.

Inflammatory activators such as demodex infestation may play a role in the pathogenesis of some cases of androgenetic alopecia that do not respond to common treatments such as minoxidil and finasteride. Thus, we tested that RP measured PGE_2 and nitric oxide levels using macrophage cell line RAW 264.7 cells. Treatment with LPS significantly increased PGE_2 (by 19.55 ± 0.54%) and nitric oxide (by 11.97 ± 0.28%) compared with the control, while treatment with RP and AA reduced LPS-induced PGE_2 (by 13.50 ± 0.59% to 9.34 ± 0.23% and 14.56 ± 0.47% to 9.96 ± 0.27%) and nitric oxide (by 11.06 ± 0.16% to 5.60 ± 0.12% and 9.73 ± 0.27% to 4.79 ± 0.29%) (Figure 3A, B, C). According to our results, RP had a significantly greater hair growth-promoting effect

than that exerted by its main component, AA, *in vivo*. These data demonstrate that RP extract and AA are effective in hair growth effects in animals and thus it may be useful for treatment for alopecia.

4. Conclusion

The present study showed that RP and its main constituent AA promoted hair growth in an alopecia mouse model. Further, it was previously reported to inhibit 5 α-reductase activity. Therefore, this study provides useful *in vivo* evidence that RP and its main constituent AA exhibited significant hair growth-promoting potential and suggests that these substances can be applied as hair loss treatments.

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