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Complete List of Authors:	Kwon, Tae-Rin; Chung-Ang University College of Medicine, Department of Dermatology Oh, Chang Taek; Chung-Ang University College of Medicine, Department of Dermatology Park, Hye Min; Green Cross Corporation, Han, Hae Jung; Green Cross Corporation, Ji, Hyi Jeong; Green Cross Corporation, Kim, Beom Joon; Chung-ang University College of Medicine, Department of Dermatology
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Potential Synergistic Effects of Human Placental Extract and Minoxidil on Hair Growth-Promoting Activity in C57BL/6J Mice

Tae-Rin Kwon[1,2*], Chang Taek Oh[2], Hye Min Park[3], Hae Jung Han[3], Hyi Jeong Ji[3], Beom Joon Kim[1,2†]

[1]Department of Medicine, Graduate School, Chung-Ang University, Seoul, Korea

[2]Department of Dermatology, Chung-Ang University College of Medicine, Seoul, Korea

[3]Green Cross Corporation, 303 Bojeong-dong, Giheung-gu, Yongin, Korea

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Corresponding author:

Beom Joon Kim, MD, Department of Dermatology, Chung-Ang University Hospital, 102 Heukseokro, Dongjak-gu, Seoul 156-755, South Korea

E-mail: beomjoon74@gmail.com., Tel: 82-2-6299-1525, Fax: 82-2-823-1049

ABSTRACT

Background Human placenta extract (HPE) has been used for alleviation of fatigue, wound healing, and antiaging functions; however, it has not yet been studied in hair growth.

Aim In the present study, we evaluated the *in vitro* effect of HPE on hair growth by observing its actions on human dermal papilla cells (DPCs). The purpose of this research was to define HPE promotion of induction of anagen hair growth in telogen using C57BL/6J mice and to understand the synergistic molecular mechanisms of HPE and minoxidil (MXD) actions on hair growth.

Methods We examined the effects of HPE and MXD on C57BL6/J mice using hematoxylin and eosin (H&E) staining, quantitative histomorphometry, hair growth scoring, immunohistochemistry, and immunofluorescence on the dorsal skins of C57BL/6J mice**.**

Results We found that HPE synergistically augmented the effects of MXD, a promoter of hair growth. In particular, histomorphometric analysis data indicated that subcutaneous injection of HPE induced an earlier anagen phase and prolonged the anagen phase and also stimulated increases in both the number and size of hair follicles in groups treated with HPE and HPE plus MXD.

Conclusions From our data, we conclude that HPE increases β-catenin and Wnt3a expression levels. Overall, our findings suggest that HPE in combination with MXD has hair growth-promoting activity and is a potential novel therapeutic treatment for alopecia or baldness in humans.

INTRODUCTION

Dysregulation of the hair growth cycle has been shown to be associated with hair loss or

alopecia. Hair loss is considered to be an independent disease that can be accompanied by physical, psychological, or social problems. There are various causes of hair loss including aging, hormone imbalance, stress, and nutritional deficiency, which can cause hair loss in both men and women [1].

The number of patients suffering from hair loss or alopecia has increased dramatically in recent years. Minoxidil is the most effective treatment for hair loss and has been approved by the Food and Drug Administration (FDA) [2]. However, the efficacy of minoxidil is limited and transient due to unpredictable results and side effects, such as resumption of hair loss after discontinuing use [3]. Therefore, there is a pressing need to develop alternative hair loss treatments [4]. Recently, it was reported that injection of platelet-rich plasma (PRP) into mice induces a hair growth effect [5], indicating that PRP helps to maintain the hair and scalp and stimulates activity, thereby encouraging hair growth.

In an effort to develop new therapies to enhance hair growth, we screened human placental extract (HPE) because it contains a variety of growth factors, cytokines, and other physiologically-active substances such as PRP. Human placental extract has been approved by the FDA for use in humans and is now being used widely for improvement of fatigue [6,7]. Studies using animal models have provided evidence for liver function improvement [8] and wound healing [9] attributes of HPE as assessed via liver regeneration and production of TGF-beta and VEGF, respectively. However, despite the recent popularity of HPE, its mechanism is not yet fully understood. Importantly, there is no scientific report or verification of HPE efficacy in the treatment of human hair growth. Hence, the present study was undertaken to evaluate the effect of HPE or HPE in combination with MXD on hair growth-promoting effects.

MATERIALS AND METHODS

Human dermal papilla isolation and culture

Human dermal papilla cells (hDPCs) were purchased from Cefobio (Seoul, Korea) as primary cells and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco-BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS; Invitrogen-Gibco-BRL) and 1% penicillin in a humidified environment. Human follicular DPCs in the third or fourth passages were used.

Cell proliferation assay

hDPCs were plated at a density of 1.5×10^4 cells/well in 96-well plates, and the proliferation of the hDPCs was measured using a CCK-8 assay (Dojindo, Rockville, MD, USA). After treatment, the cells were continuously cultured for 96 h under the appropriate experimental conditions. CCK-8 solution (10 μl) was added to the cells in 1 ml DMEM and incubated for 2 h at 37°C, and the absorbance was measured at 450 nm using a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Hair growth activity in vivo

A total of 56 female C57BL/6J mice (six weeks old) were purchased from Japan SLC (Shizuoka, Japan) and divided randomly into seven groups (eight mice/group): Normal, non-treated; Vehicle, received subcutaneous injection of saline after topical application of ethanol; 2% MXD (Minoxidil 2g/100 ml, Dong Kwang Pharm Co., Ltd., Korea), received subcutaneous injection of saline after topical application of MXD; 2% HPE, received subcutaneous injection of HPE (2%) after topical application of ethanol; 20% HPE, received subcutaneous injection of HPE (20%) after topical application of ethanol; 100% HPE,

received subcutaneous injection of HPE (100%) after topical application of ethanol; HPE plus MXD, received subcutaneous injection of HPE (100%) after topical application of 2% MXD). Anagen was induced by depilation of skin on the backs of C57BL/6 mice that were in the telogen phase of the cycle, as previously described [10]. The hydrolysate of human placenta (HPE) was provided by Green Cross Japan Bio Products (Laennec: GCJBP Corporation, Yongin, Korea). On day 1, each mouse received a topical treatment with either 200 ul of ethanol or MXD (2%) followed by a subcutaneous quad injection in the upper back with 100 µl (total 400 µl) saline or HPE (2, 20, or 100%). Topical MXD treatments were continued on the dorsal skin of treated animals every two days for 21 days; control animals received the vehicle solution alone. The back of each mouse was observed and photographed every other day following depilation. All experiments lasted for 21 days, and the mice were then sacrificed. All procedures involving animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung Ang University in Korea (IRB Number: 13-0002).

Method for determining hair growth promoting activity

The hair growth-promoting activities of substances were measured by the darkening of the dorsal skin, which indicated the anagen phase of hair follicle growth. Hair growth was measured at 0, 6, 9, 12, 15, 18, and 21 days by assigning a hair growth score as follows: 0 = no growth observed; 1 = up to 20% growth; 2 = 20–40% growth; 3 = 40–60% growth; 4 = 60–80% growth; and 5 = 80% to full growth observed. Digital images were analyzed for total hair growth and follicle count at day 21 by quantification of hair growth area using Image-Pro Plus software Version 7.0 (Media Cybernetics, Silver Spring, MD, USA).

Quantitative histomorphometry

Dorsal skin biopsies were fixed with 4% paraformaldehyde (PFA) for routine histology, paraffin-embedded, and processed for hematoxylin-eosin staining. Individual hair follicles were confined to specific hair cycle stages (telogen or anagen I–VI), following the classification of Chase [11].

Histological examination

Dorsal tissues were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin, and 5-µm-thick sections were transferred to probe-on-plus slides (Fisher Scientific, Pittsburg, PA, USA). De-paraffinized skin sections were stained with H&E. Some sections were stained for immunohistochemical markers using monoclonal antibodies against β-catenin (1:500, 610154, BD Transduction, Lexington, KY, USA) or Wnt3a (1:500, ab28472, Abcam, Cambridge, MA, USA). Immunohistochemical analysis was performed using a high-temperature antigen unmasking technique. The sections were heated in an unmasking solution (citrate buffer, pH 6.0), washed, and then incubated with mouse primary monoclonal antibodies at room temperature for 1 h. This procedure was followed by incubation with secondary antibodies (Envision Detection kit K5007, DAKO, Glostrup, Denmark). The reaction products were developed with diaminobenzidine solution as a chromogen, and the sections were rinsed and counterstained with hematoxylin. After rinsing, the sections were dehydrated, covered with permount (Fisher Scientific, Fair Lawn, NJ, USA), and examined by light microscopy to assess the histological changes. Two independent blinded observers evaluated the serial sections.

Statistical analysis

Data are presented as mean ± SEs, and statistical comparisons between treated groups and the untreated group were performed using one-way ANOVA analyses followed by post-hoc Tukey's test for direct comparison between specific groups. The results are expressed as the mean ± standard deviation of at least three independent experiments, and P values $p<0.05$: *, $p<0.01$: **, and $p<0.001$: *** were considered statistically significant.

RESULTS

Human placental extract with MXD dramatically enhanced viability of human DPCs

To compare the effects of HPE and MXD on proliferation of hDPCs, a CCK-8 assay was performed. Cells were treated with various concentrations of HPE (0, 10, and 20%) and MXD (0, 0.5, and 1 μM) for 96 h. When hDPCs were treated for 96 h, the proliferation rate increased by 42% with 20% HPE and by 28% with 0.5 μM MXD. Furthermore, both HPE and MXD substantially increased cell viability in a dose-dependent manner (Fig. 1). Interestingly, cell viability was significantly increased in a dose-dependent manner when cells were treated with a combination of HPE and 0.5 μM MXD compared to 0.5 μM MXD treatment alone. Therefore, we hypothesized that the combined treatment of HPE plus MXD synergistically increased proliferation of hDPCs.

Human placental extract enhanced hair growth with MXD in the C57BL/6 mouse model

To evaluate the effects of activated HPE on hair growth *in vivo*, we divided mice into seven randomized groups (n=8). Darkening of the dorsal skin was observed after the every-other-day application of HPE alone. On day 8 after depilation, the skin color in the center region in the HPE plus MXD group was darker than the same area in the 2% MXD group (Fig. 2). The HPE plus MXD group showed skin darkening earlier than the HPE-alone groups. On day 21

after depilation, the back skin of all mice was in mature anagen phase. Importantly, HPE affected hair growth to influence hair cycling in C57BL6/J mice (Fig. 3A). We also evaluated the hair growth score of mice treated with HPE or HPE plus MDX at days 0, 6, 9, 12, 15, 18, and 21. We found that the hair growth score was significantly increased in the HPE plus MXD group (2% MXD with 20% HPE) compared to the Normal group on 21 day (Fig. 3B). Additionally, the weights of newly grown hairs in all the test groups were measured and compared with that of the control group. The weight of newly grown hair was highest for the HPE plus MXD group (data not shown). These results suggest that treatment with HPE plus MXD may induce hair growth by promoting activity through early telogen to anagen conversion of hair follicles in C57BL/6 mice compared to 2% MXD treatment alone.

Effects of human placental extract on the formation of hair follicles

H&E-stained skin tissues obtained 21 days after depilation were used to observe the status of hair follicles and hair growth by optical microscopy. On day 21, the normal group demonstrated partial, weak development of hair roots and areas deficient in inner root sheath. In comparison, the HPE-treated groups had well-developed hair follicles and inner root sheaths, as well as increased hair growth (Fig. 4A). The number of follicles in the anagen (A) phase, catagen, or telogen (T) phase were counted, and the A/T ratio was determined. The anagen/telogen (A/T) ratio was increased in the HPE-treated and HPE plus MXD groups (Fig. 4B). The combined HPE and MXD-treated groups were in anagen phase VI, whereas the vehicle group remained in anagen phase III. As a result, the A/T ratios for the 20% HPE group (Tukey's test, $p < 0.05$) and the HPE plus MXD group (Tukey's test, $p < 0.05$) were higher than the ratio for the Normal group. As shown in Fig. 4C, skin thicknesses were measured over the total length of the hair, starting in the deep subcutis layer during the

anagen phase [12]. We found that thickness varied with increasing distance from the follicle, and skin thickness was significantly increased in the HPE plus MXD group ($P < 0.001$) compared to the Normal group. In addition, the HPE-treated, HPE plus MXD, and 2% MXD groups also exhibited significantly increased numbers of hair follicles compared to the Normal group; however, no differences were observed between the HPE-treated and HPE plus MXD groups (Figure 4D). Histologic study indicated that the HPE-treated groups had markedly increased depth and were more often in the anagen stage of hair growth compared with the Normal group. Therefore, HPE appeared to effectively promote hair growth and prolong the mature anagen phase in our animal model.

Effects of human placental extract on the development and number of mouse hair follicles

To analyze the effect of HPE on induction of hair growth, we prepared horizontal sections and measured the number and size of hair follicles. Day 21 dorsal skin biopsies were collected, and the segments were serially and horizontally sectioned by microtome in the directions of the upper and lower dermis. The sections showed hairs from the isthmus to the bulbar portion, which allowed for the differentiation of vellus, catagen, and telogen hairs [13, 14]. We quantified the area of hair growth using Image-Pro Plus software. As shown in Fig. 5A, the hair follicle counts were significantly increased in the HPE plus MXD group (Tukey's test, $P < 0.05$) compared to the 2% MXD group. The mean hair follicle counts for the horizontal sections of the HPE plus MXD group and 2% MXD group were 139.75 ± 6.70 and 112.63 ± 6.87, respectively (Fig. 5b). These results may suggest that HPE stimulated early embryonic development of hair and also that HPE has significant effects on hair follicle development.

Analysis of β-catenin and Wnt3a expression in the skin of C57BL/6J mice

To evaluate the signaling mechanism underlying the induction of anagen phases in the HPE and HPE plus MXD groups, we performed immunohistochemistry and immunofluorescence microscopy on dorsal skin specimens using antibodies specific to β-catenin and Wnt3a. Animals treated with HPE exhibited anagen induction as evidenced by positive staining for β-catenin (Figs. 6a, b, upper panel) and Wnt3a (Figs. 6a, b, down panel). Immunohistochemistry analysis provided further support for increased expression levels of β-catenin and Wnt3a. Specifically, immunofluorescence revealed strong depositions of β-catenin and Wnt3a around hair follicles. In addition, the HPE plus MXD group had markedly increased β-catenin and Wnt3a expression levels compared with the group treated with HPE-alone. In particular, anti-β-catenin antibody stained putative outer root sheath cells, while basophilic and potential hair matrix cells were occasionally stained. Furthermore, Wnt3a staining was positive in transitional cells and putative hair-like structures surrounding the cells, which is indicative of the formation of hair follicle placodes [15]. These results demonstrate that HPE activates certain functions in the anagen phase, and that HPE activates hair follicle development through the Wnt/β-catenin pathway [16]. Taken together, these findings suggest a possible role for HPE in the hair-promoting activity related to hair morphogenesis.

DISCUSSION

The number of patients suffering from hair loss and alopecia has increased dramatically in recent years [17]. There are various causes of hair loss, including aging, hormone imbalance, stress, and nutritional deficiency, all of which can cause hair loss in both men and women [18, 19]. Two drugs are currently licensed for the treatment of male androgenetic alopecia, namely,

oral finasteride [20] and topical minoxidil solution [21]. However, the efficacies of these two medications are limited and transient due to unpredictable efficacies, side effects, and resumption of hair loss upon discontinuation. In the search for potential alternative candidate agents for the treatment of hair loss [22], human placental extract (HPE), a potent hair growth modulator, was investigated as a promising candidate using *in vitro* and *in vivo* studies. HPE has been approved and is now widely used for alleviation of fatigue, skin whitening, and anti-aging applications [6]. Interestingly, a previous report showed that cow placenta extract (CPE) can promote hair growth in C57BL/6 mice by elongating hair shafts and increasing hair follicle number [23].

Several reports have shown that growth factors from the area surrounding the hair follicle stimulate hair growth in animal models [24]. Interestingly, human placenta extracts contain a large variety of growth factors, cytokines, and other physiologically-active substances. In the present study, we tested the possibility that HPE can stimulate hair growth-promoting effects in vitro and in vivo. Our findings are expected to lead to the development of new alternative medicines for the treatment of hair loss-related disorders, especially androgenic alopecia.

Cell viability was significantly dose-dependently increased by a combination treatment of HPE and 0.5 μM MXD compared to the 10% HPE treatment group. However, the mechanism by which HPE enhances proliferation remains to be elucidated. As shown in Fig. 2, animals were shaved, and skin color was evaluated in order to determine changes in hair growth. Beginning on day 8 after depilation, the skin color of the center region in the HPE plus MXD group was darker than the 2% MXD group. Likewise, the HPE plus MXD group showed earlier darkening of skin than the other groups of C57BL6/J mice, indicating earlier hair growth in this group. We also evaluated the effect of HPE on hair growth by determining the hair growth score every other day for 21 days. Our results suggested that the effects of HPE

plus MXD treatment on hair growth-promoting activity was mediated by early telogen-to-anagen conversion of hair follicles in C57BL/6 mice.

H&E-stained skin tissues from 21 days after depilation were used to observe the status of hair follicles and hair growth. Hair thickness was measured during the anagen phase along the total length of each hair starting in the deep subcutis layer. Histologic studies showed that the HPE-treated group had markedly increased depth and more anagen-stage hair follicles compared with the Normal group, suggesting that HPE effectively promoted hair growth and prolonged the mature anagen phase in our animal model.

To measure the number and size of the hair follicles induced by HPE, horizontal sectioning was performed. At day 21, dorsal skin biopsies were collected, and each segment was sectioned serially and horizontally using a microtome in the direction of the upper and lower dermis. Hair follicle counts were significantly increased by HPE treatment, which indicates that HPE may stimulate early embryonic developmental processes and play a significant role in hair follicle development.

Wnt/β-catenin signaling promotes the development of new hair follicles and is required for initiation of hair morphogenesis [25, 26, 27]. Within established hair follicles, Wnt cascade signaling plays a key role in the activation of bulge stem cells in order to stimulate hair formation, and this signaling is relayed by β-catenin and Lef1 [28]. To explore the molecular mechanisms by which Wnt/β-catenin signaling regulates hair follicle formation [29], we used immunohistochemistry and immunofluorescence to visualize β-catenin and Wnt3a in the hair shafts of C57BL6/J mice. Immunostaining studies showed that the HPE plus MXD group had markedly increased expression of β-catenin and Wnt3a compared with the group treated with HPE alone. These results indicated that the effects of HPE were related to certain functions in the anagen phase, and that HPE activated hair follicle development through the Wnt/β-

catenin pathway. Specifically, it is possible that HPE stimulated the hair follicles to enter into the anagen phase, and that the elongation of the immature hair follicles was accelerated by HPE. In addition, treatment with HPE or HPE plus MXD promote hair follicle elongation in humans, thus extending the anagen phase. This suggests its usefulness in the potential treatment of male pattern baldness. However, further studies are necessary to determine the possible side effects of HPE treatment and whether they are different from those of MXD treatment in humans.

Overall, we demonstrated that HPE stimulates the growth of derma papilla cells and promotes prolonged anagen phase of hair growth in C57BL6/J mice. Taken together, our findings suggest that HPE may stimulate the early embryonic development process and play a significant role in hair follicle development. Further *in vitro* and *in vivo* studies of the bioactive components in HPE will increase our understanding of the mechanisms by which it stimulates hair growth. Moreover, additional experiments will be required to validate the results presented in this study.

CONCLUSION

In conclusion, we demonstrated that HPE improves hair-promoting activity, probably through mechanisms acting via the Wnt/β-catenin pathway, thereby improving hair growth in a C57BL/6J mouse model. Our findings are expected to lead to the development of a potential candidate combination therapy for hair-loss patients.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest are disclosed.

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FIGURE LEGENDS

Figure 1. Effects of human placental extract (HPE) on viability of human dermal papilla cells. Cells were treated with HPE, MXD, or both for 96 h. Cells were treated with various concentrations of HPE (0, 5, 10, 20 %) or MXD (0, 0.1, 0.5, 1 μM) for 96 h. Cell viability was measured with the CCK-8 assay. Results are expressed as fold changes (mean ± S.E.M) relative to the control. *P < 0.05, **P < 0.01, compared with the control. #P < 0.05, ##P < 0.01, ###P < 0.001, compared with 0.5 μM MXD treatment. HPE; human placental extract, MXD; minoxidil

Figure 2. Comparison of hair growth in C57BL/6J mice. The back skins of treated mice were photographed at 0, 8, 15, and 21 days after depilation (Canon 3000D). The back skin of each seven-week-old male C57BL/6J mouse (n=8) was shaved, treated with a topical application of 200 μl of ethanol or MXD (2%), and then injected subcutaneously on the center upper back with quad injections of 100 μl (total 400 μl) saline or HPE (2, 20, or 100%); topical MXD or ethanol treatments were repeated on the dorsal skin of each animal every other day for 21 days. Seven experimental groups were formed using three parameters, as described in the *Materials and Methods*.

Figure 3. Hair growth-promoting effects of human placental extract and MXD in the C57BL/6J mouse model. (a) Photometric comparison of hair growth of a shaved C57BL/6J

mouse after 6, 9, 12, 15, 18, and 21 days. (b) Hair growth scores were evaluated using a scoring index as follows: 0 = no growth observed; 1 = up to 20% growth; 2 = 20–40% growth; 3 = 40–60% growth; 4 = 60–80% growth; and 5 = 80% to full growth observed. Quantification of hair growth area was performed using Image-Pro Plus software (n = 8). Values shown are mean ± S.E.M (n = 8/mouse; *p < 0.05, **p < 0.01 and ***P < 0.001, vs. Normal).

Figure 4. The effects of HPE and MXD on hair follicle formation in C57BL/6J mice. (a) The effects of HPE and MXD on hair follicles in telogen stage were analyzed using hematoxylin-eosin (H&E) staining (upper, longitudinal section; lower, transverse section). Comparison of histological images of hair follicles. A representative histology result from eight animals is shown. (b) Hair growth patterns (anagen/telogen ratio) in C57BL/6 mice. Values are mean ± S.E.M (n = 8/mouse; *p < 0.05, **p < 0.01 and ***P < 0.001, vs. Normal). (c) Histograms of hair follicle counts in transverse sections. (d) Skin thickness is defined as the distance from the epidermal granular layer to the top edge of the panniculus camosus. Original magnification: × 200. Values are mean ± S.E.M (n = 8/mouse; *p < 0.05, **p < 0.01 and ***P < 0.001, vs. Normal).

Figure 5. The effects of HPE and MXD on hair follicle number in C57BL/6J mice. The skin was fixed in 10% buffered formalin, and longitudinal slices were processed for routine histology using hematoxylin and eosin (H&E) staining. (a) The segment was sectioned serially and horizontally by microtome (4-um-thick slices) in the direction of the upper dermis (superior segment) and lower dermis (inferior segment). Each slide contained three sections, all at different levels within the specimen. This sectioning allowed us to examine the

hairs from the isthmus to the bulbar portion and thereby differentiate vellus, catagen, and telogen hairs. Values shown are mean ± S.E.M (n = 8/mouse; ***P < 0.001, vs. Normal) (b) Histograms of hair follicle counts in horizontal sections. Values shown are mean ± S.E.M (n = 8/mouse; ***P < 0.001, vs. Normal, #P < 0.05, vs. 2% MXD.).

Fig 6. Representative sections of beta-catenin and Wnt3a immune-reacted skin tissues of C57BL/6J mice. (a) Dorsal skin biopsies were taken after 21 days and were immunohistochemically stained. The beta-catenin+/Wnt3a+ areas were stained by DAB (red arrow; strong expression), with a hematoxylin counterstain. (b) Immunofluorescence staining for β-catenin and Wnt3a (red, β-catenin; green, Wnt3a). Nuclei were counterstained with DAPI (blue), n=8 mice per group. Original magnification: × 200.

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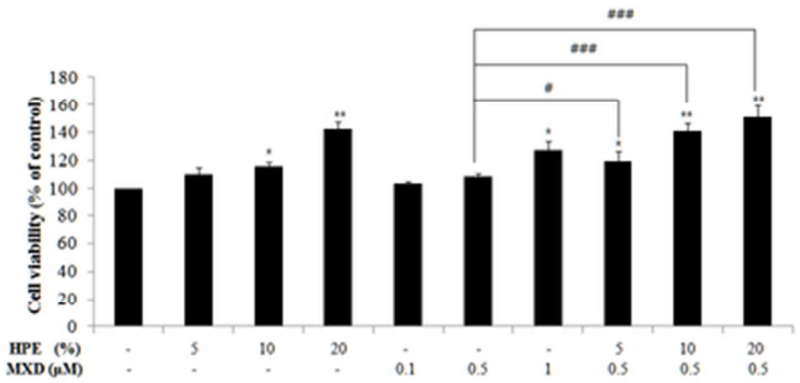

Fig 1. Effects of human placental extract (HPE) on viability of human dermal papilla cells. Cells were treated with HPE, MXD, or both for 96 h. Cells were treated with various concentrations of HPE (0, 5, 10, 20 %) or MXD (0, 0.1, 0.5, 1 μM) for 96 h. Cell viability was measured with the CCK-8 assay. Results are expressed as fold changes (mean ± S.E.M) relative to the control. *P < 0.05, **P < 0.01, compared with the control. #P < 0.05, ##P < 0.01, ###P < 0.001, compared with 0.5 μM MXD treatment. HPE; human placental extract, MXD; minoxidil
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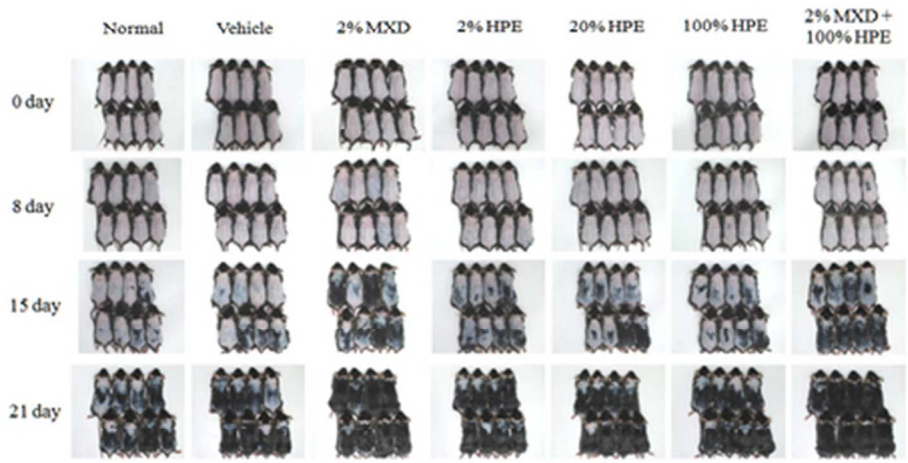

Fig 2. Comparison of hair growth in C57BL/6J mice. The back skins of treated mice were photographed at 0, 8, 15, and 21 days after depilation (Canon 3000D). The back skin of each seven-week-old male C57BL/6J mouse (n=8) was shaved, treated with a topical application of 200 μl of ethanol or MXD (2%), and then injected subcutaneously on the center upper back with quad injections of 100 μl (total 400 μl) saline or HPE (2, 20, or 100%); topical MXD or ethanol treatments were repeated on the dorsal skin of each animal every other day for 21 days. Seven experimental groups were formed using three parameters, as described in the Materials and Methods.
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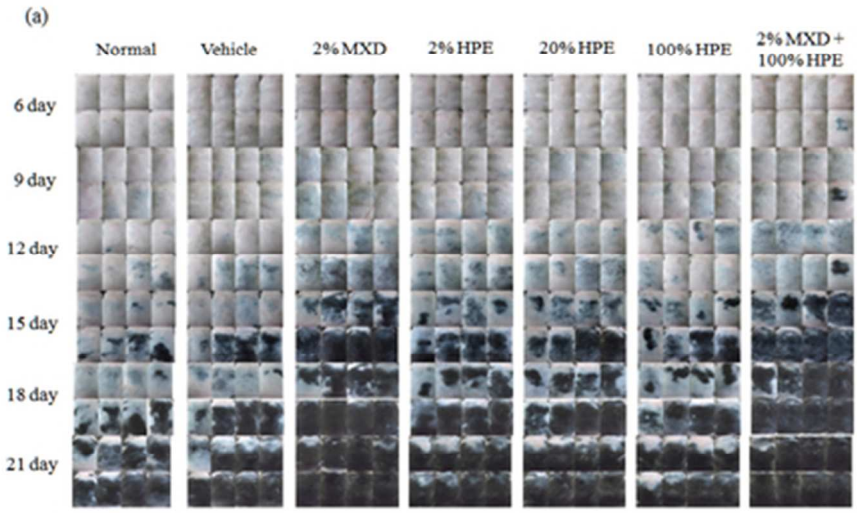
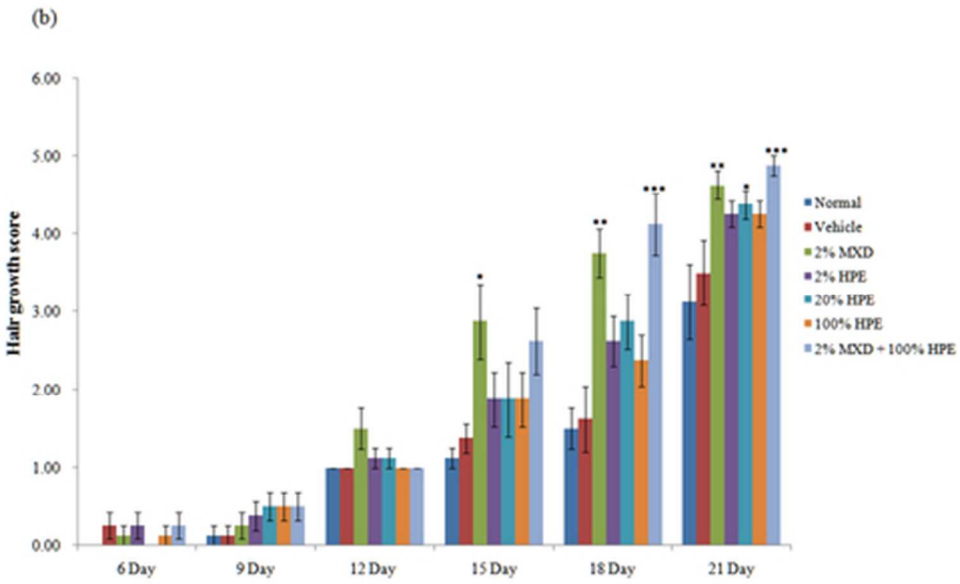


Fig 3. Hair growth-promoting effects of human placental extract and MXD in the C57BL/6J mouse model. (a) Photometric comparison of hair growth of a shaved C57BL/6J mouse after 6, 9, 12, 15, 18, and 21 days. 42x42mm (300 x 300 DPI)



(b) Hair growth scores were evaluated using a scoring index as follows: 0 = no growth observed; 1 = up to 20% growth; 2 = 20–40% growth; 3 = 40–60% growth; 4 = 60–80% growth; and 5 = 80% to full growth observed. Quantification of hair growth area was performed using Image-Pro Plus software (n = 8). Values shown are mean ± S.E.M (n = 8/mouse; *p < 0.05, **p < 0.01 and ***P < 0.001, vs. Normal).
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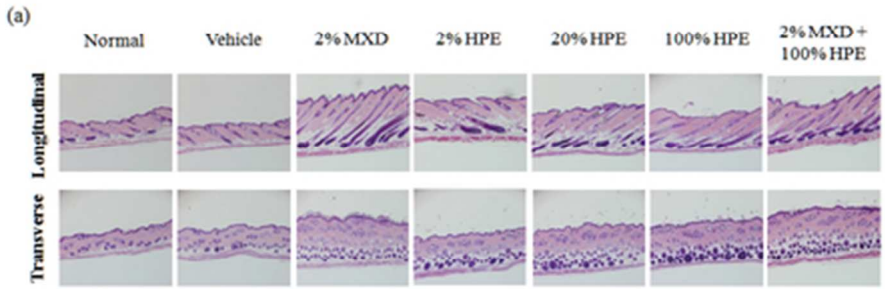

Figure 4. The effects of HPE and MXD on hair follicle formation in C57BL/6J mice. (a) The effects of HPE and MXD on hair follicles in telogen stage were analyzed using hematoxylin-eosin (H&E) staining (upper, longitudinal section; lower, transverse section). Comparison of histological images of hair follicles. A representative histology result from eight animals is shown.
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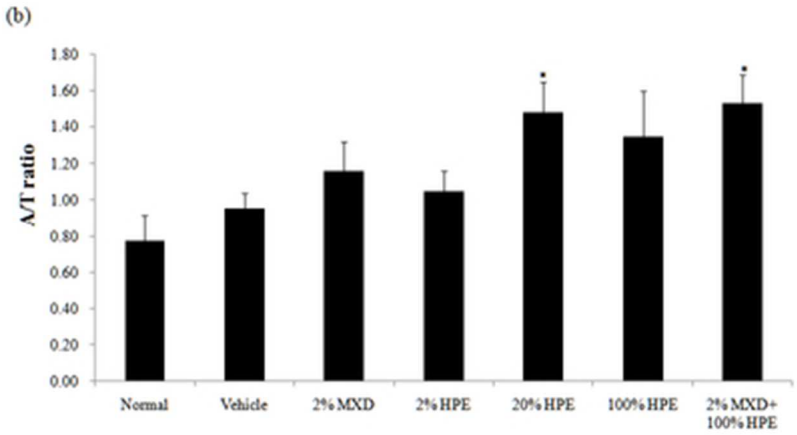
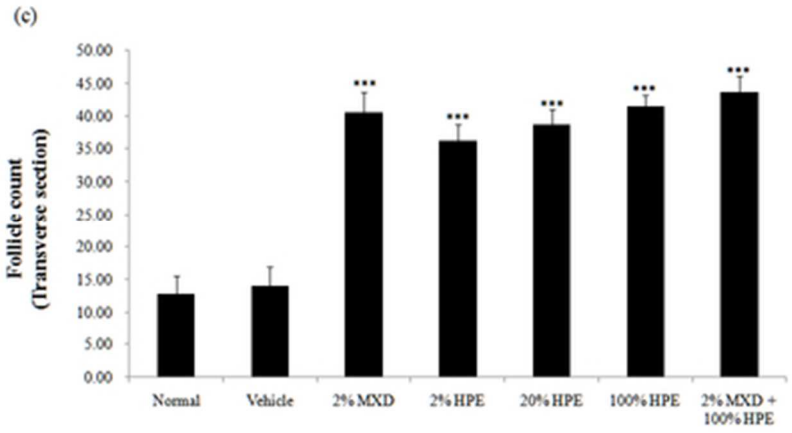


(b) Hair growth patterns (anagen/telogen ratio) in C57BL/6 mice. Values are mean ± S.E.M (n = 8/mouse; *p < 0.05, **p < 0.01 and ***P < 0.001, vs. Normal).
38x38mm (300 x 300 DPI)



(c) Histograms of hair follicle counts in transverse sections.
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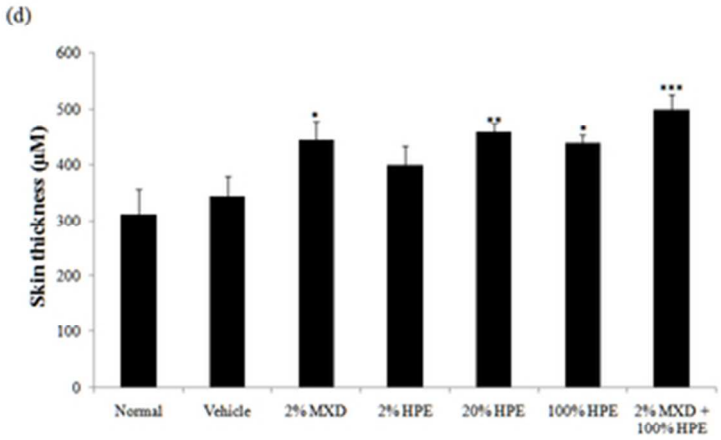

(d) Skin thickness is defined as the distance from the epidermal granular layer to the top edge of the panniculus camosus. Original magnification: × 200. Values are mean ± S.E.M (n = 8/mouse; *p < 0.05, **p < 0.01 and ***P < 0.001, vs. Normal).
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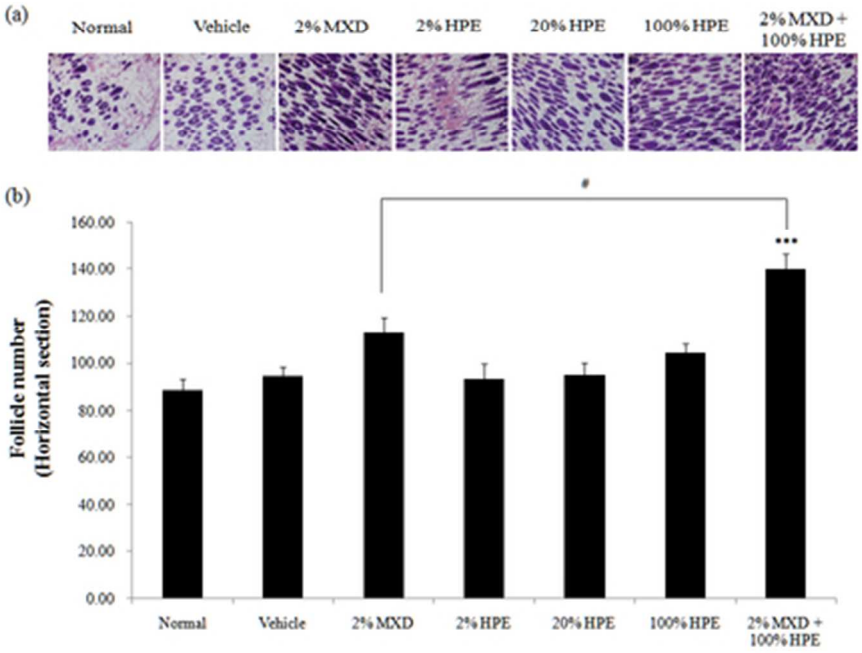

Figure 5. The effects of HPE and MXD on hair follicle number in C57BL/6J mice. The skin was fixed in 10% buffered formalin, and longitudinal slices were processed for routine histology using hematoxylin and eosin (H&E) staining. (a) The segment was sectioned serially and horizontally by microtome (4-um-thick slices) in the direction of the upper dermis (superior segment) and lower dermis (inferior segment). Each slide contained three sections, all at different levels within the specimen. This sectioning allowed us to examine the hairs from the isthmus to the bulbar portion and thereby differentiate vellus, catagen, and telogen hairs. Values shown are mean ± S.E.M (n = 8/mouse; ***P < 0.001, vs. Normal) (b) Histograms of hair follicle counts in horizontal sections. Values shown are mean ± S.E.M (n = 8/mouse; ***P < 0.001, vs. Normal, #P < 0.05, vs. 2% MXD.).
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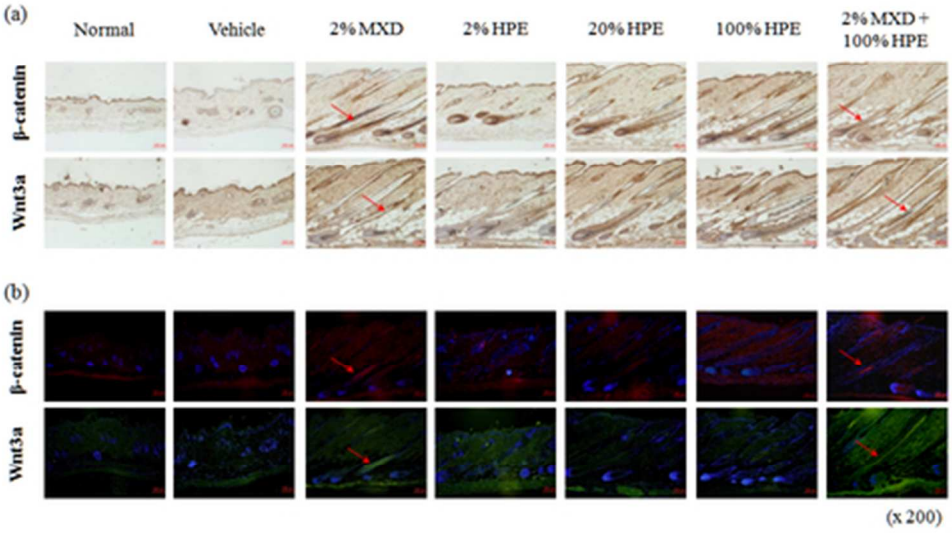

Fig 6. Representative sections of beta-catenin and Wnt3a immune-reacted skin tissues of C57BL/6J mice. (a) Dorsal skin biopsies were taken after 21 days and were immunohistochemically stained. The beta-catenin+/Wnt3a+ areas were stained by DAB (red arrow; strong expression), with a hematoxylin counterstain. (b) Immunofluorescence staining for β-catenin and Wnt3a (red, β-catenin; green, Wnt3a). Nuclei were counterstained with DAPI (blue), n=8 mice per group. Original magnification: × 200.
42x42mm (300 x 300 DPI)