POTENTIAL SYNERGISTIC EFFECTS OF HUMAN PLACENTAL EXTRACT AND MINOXIDIL ON HAIR GROWTH-PROMOTING ACTIVITY IN C57BL/6J MICE

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Summary

Background
Human placenta extract (HPE) has been used to alleviate tiredness and promote wound healing, and for its antiageing functions; however, it has not yet been studied for its effects on hair growth. 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).

Aim
To define how HPE promotes induction of anagen hair growth during the telogen phase, 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 haematoxylin and eosin 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. It also stimulated increases in both the number and size of hair follicles in groups treated with HPE alone and HPE + MXD.

Conclusions
From our data, we conclude that HPE increases $\beta$-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 a distinct condition that can be accompanied by physical, psychological or social problems. There are various causes, including ageing, hormone imbalance, stress and nutritional deficiency, which can cause hair loss in both men and women.[1] The number of patients reporting hair loss or alopecia has increased dramatically in recent years. Minoxidil (MXD) is the most effective treatment for hair loss, and has been approved by the US Food and Drug Administration (FDA).[2] However, the efficacy of is MXD is limited and transient, because of its unpredictable results and side effects, such as resumption of hair loss after discontinuing use.[3] Therefore, alternative hair loss treatments are needed.[4] Recently, it was reported that injection of platelet-rich plasma (PRP) into mice induces hair growth, indicating that PRP helps to maintain the hair and scalp, and stimulates hair follicle 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.[5] HPE has been approved by the FDA for use in humans, and is now being used widely for treatment of tiredness.[6, 7] Studies using animal models have provided evidence that HPE has liver function improvement[8] and wound healing[9] attributes, as assessed via liver regeneration and production of transforming growth factor-$\beta$ and vascular endothelial growth factor, respectively. However, despite the recent popularity of HPE, its mechanism is not yet fully understood. Importantly, there has been 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 on hair growth of HPE alone or in combination with MXD.
Methods

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 (institutional review board no. 13-0002).

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

Cell proliferation assay
hDPCs were plated at a density of $1.5 \times 10^4$ cells/well in 96-well plates, and their proliferation was measured using a cholecystokinin (CCK)-8 assay (Dojindo, Rockville, MD, USA). Cells were treated with various concentrations of HPE (0%, 10% and 20%) and MXD (0, 0.5 and 1 \( \mu \text{mol/L} \)) for 96 h. CCK-8 solution (10 \( \mu \text{L} \)) was added to the cells in 1 mL DMEM, and incubated for 2 h at 37 °C. Absorbance was measured at 450 nm using a microplate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA).

Hair growth activity in vivo
In total, 56 female C57BL/6J mice, 6 weeks old, (Japan SLC, Shizuoka, Japan) were divided randomly into 7 groups (8 mice/group). Group 1 comprised normal (untreated) mice, while group 2 (vehicle) received topical application of ethanol, followed by subcutaneous (SC) injection of saline. Group 3 was the MXD-only group, which was treated with topical application of 2% MXD (minoxidil 2 g/100 mL; Dong Kwang Pharm Co. Ltd., Seoul, Korea), followed by SC injection of saline. Groups 4–6 were the HPE-only groups, which were treated with topical application of ethanol, followed by SC injection of 2%, 20% or 100% HPE [Laennec: Green Cross Japan Bio Products (GCJBP) Corp., Yongin, Korea], respectively. Finally, group 7 (HPE + MXD), received topical application of 2% MXD, followed by SC injection of 100% HPE.

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] On day 1, each mouse received topical treatment with either 200 \( \mu \text{L} \) of ethanol or 2% MXD, followed by 4 \( \times \) 100 \( \mu \text{L} \) SC injections of saline or HPE (2%,
20%, or 100%) in the upper back as detailed above. Topical MXD treatments were continued on the dorsal skin of treated animals every 2 days for 21 days; control animals received the vehicle solution alone. The back of each mouse was examined and photographed (3000D; Canon Inc., Tokyo, Japan) every other day following depilation. All experiments lasted for 21 days, and the mice were then euthanized.

Determination of 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 on days 0, 6, 9, 12, 15, 18 and 21 by assigning a hair growth score (0 = no growth, 1 = up to 20% growth, 2 = 20–40% growth, 3 = 40–60% growth, 4 = 60–80% growth and 5 = 80–100% growth) Digital images were analysed for total hair growth and follicle count on day 21 by quantification of hair growth area using Image-Pro+ software (v7.0; Media Cybernetics, Silver Spring, MD, USA).

Quantitative histomorphometry
Dorsal skin biopsies were fixed with 4% paraformaldehyde (PFA) for routine histology, embedded in paraffin wax, and processed for haematoxylin and eosin (H&E) staining. Individual hair follicles were categorized into specific hair cycle stages (telogen or anagen I–VI), following the classification of Chase.[11]

Histological examination
Dorsal skin biopsies were collected, fixed with 4% paraformaldehyde (PFA) and embedded in paraffin wax. The segments were cut by microtome into serial horizontal sections 5 μm thick, in the directions of the upper and lower dermis. The sections were transferred to pretreated slides (ProbeOn Plus™; Fisher Scientific, Pittsburgh, PA, USA), dewaxed, and stained with H&E. Some sections were stained for immunohistochemical markers using monoclonal antibodies against β-catenin (1 : 500 dilution; cat. no. 610154; BD Transduction, Lexington, KY, USA) or Wnt3a (1 : 500; cat. no. 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 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 (DAB) solution as chromogen, and the sections were rinsed and counterstained with haematoxylin. After rinsing, the sections were dehydrated, covered with mounting medium (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 ± SEM, and statistical comparisons between the treated groups and the untreated group were performed using one-way ANOVA analyses followed by post hoc Tukey test for direct comparison between specific groups. The results are expressed as the mean ± SD of at least three independent experiments, and P < 0.05 was considered statistically significant.

Results
Human placental extract with minoxidil dramatically enhanced viability of human dermal papilla cells
The CCK-8 assay showed that the proliferation rate of hDPCs increased by 42% with 20% HPE and by 28% with 0.5 μmol/L MXD. Furthermore, both HPE and MXD substantially increased cell viability in a dose-dependent manner (Fig. 1). Interestingly, cell viability was also significantly increased in a dose-dependent manner when cells were treated with a combination of HPE + 0.5 μmol/L MXD compared with 0.5 μmol/L MXD treatment alone. Therefore, we hypothesize that the combined treatment of HPE + MXD synergistically increased proliferation of hDPCs.

**Figure 1.** Effects of human placental extract (HPE) on viability of human dermal papilla cells treated with various concentrations of HPE (0%, 5%, 10% or 20%), MXD (0, 0.1, 0.5 or 1 μmol/L) or both for 96 h. Cell viability was measured with the CCK-8 assay. Results are expressed as fold changes (mean ± SEM) relative to the control. *P < 0.05, **P < 0.01, compared with control; #P < 0.05, ###P < 0.001, compared with 0.5 μmol/L minoxidil (MXD) treatment.
Human placental extract enhanced hair growth with minoxidil 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 (as detailed in the 'Methods' section). Darkening of the dorsal skin was observed after alternate daily application of HPE alone. On day 8 after depilation, the skin colour in the central region of the back in the HPE + MXD group was darker than the same area in the 2% MXD group (Fig. 2). The HPE + MXD group showed skin darkening earlier than the HPE-only groups. On day 21 after depilation, the hair on the back of all mice was in mature anagen phase. Importantly, HPE affected hair growth to influence hair cycling in C57BL6/J mice (Fig. 3a).

Figure 2.
Comparison of hair growth in C57BL/6J mice, photographed at 0, 8, 15 and 21 days after depilation. The skin on the back of each 6-week-old female C57BL/6J mouse (n = 8) was shaved, treated with a topical application of 200 μL of ethanol or 2% minoxidil (MXD), and then injected in the centre of the upper back with 4 × 100 μL subcutaneous injections of saline or human placental extract (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 'Methods' section.
Figure 3.

(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 (0 = no growth, 1 = up to 20% growth, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, and 5 = 80–100%). Quantification of hair growth area was performed using Image-Pro Plus software (n = 8). Values shown are mean ± SEM. n = 8/mouse. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. normal group.

We also evaluated the hair growth score of mice treated with HPE or HPE + MXD at days 0, 6, 9, 12, 15, 18 and 21, and found that the score was significantly increased in the HPE + MXD group (2% MXD + 20% HPE) compared with the normal (untreated) group on day 21 (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 + MXD group (data not shown).

These results suggest that treatment with HPE + MXD may induce hair growth by promoting early telogen to anagen conversion of hair follicles in C57BL6/J mice compared with 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, whereas the HPE-treated groups had well-developed hair follicles and inner root sheaths, as well as increased hair growth (Fig. 4a).

(a) Normal  Vehicle  2% MXD  2% HPE  20% HPE  100% HPE  2% MXD + 100% HPE

(b) A/T ratio

(c) Follicle count (Transverse section)

(d) Skin thickness (μM)
Figure 4.
(a) Effects of human placental extract (HPE) and minoxidil (MXD) on hair follicles in telogen stage in C57BL/6J mice. A representative histology result from eight animals is shown; upper panels show longitudinal sections; lower panels show transverse sections. Haematoxylin and eosin, original magnification × 200). (b) Hair growth patterns (anagen/telogen ratio) in C57BL/6 mice. Values are mean ± SEM (n = 8 mice/group); *P < 0.05, **P < 0.01 and ***P < 0.001 vs. normal). (c) Histograms of hair follicle counts in transverse sections. (d) Skin thickness (defined as the distance from the epidermal granular layer to the top edge of the panniculus camosus). Values are mean ± SEM (n = 8/mouse; *P < 0.05, **P < 0.01 and ***P < 0.001 vs. normal).
The number of follicles in anagen, catagen or telogen phases were counted, and the anagen/telogen (A/T) ratio was determined. The A/T ratio was increased in the HPE-treated and HPE + MXD groups (Fig. 4b). The HPE + 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 test, P < 0.05) and the HPE + MXD group (Tukey test, P < 0.05) were higher than that 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 + MXD group (P < 0.001) compared with the normal group. In addition, the HPE-treated, HPE + MXD and 2% MXD groups also exhibited significantly increased numbers of hair follicles compared with the normal group; however, no differences were observed between the HPE-treated and HPE + MXD groups (Fig. 4d). Histological study indicated that the HPE-treated groups had markedly increased hair-follicle 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 analyse the effect of HPE on induction of hair growth, we measured the number and size of hair follicles in horizontal sections of skin biopsies, which showed hairs from the isthmus to the bulbar portion, allowing differentiation of vellus, catagen and telogen hairs.[13, 14] Hair follicle counts were significantly increased in the HPE + MXD group (Tukey test, P < 0.05) compared with the 2% MXD group (Fig. 5a). The mean hair follicle counts for the horizontal sections of the HPE + 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 stimulates early embryonic development of hair, and that HPE has significant effects on hair follicle development.
Figure 5.
Effects of human placental extract (HPE) and minoxidil (MXD) on hair follicle number in C57BL/6J mice, using sections of fixed and processed skin tissue. (a) Tissue was sectioned serially and horizontally by microtome (5 μm 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. (b) Histogram of hair follicle counts in horizontal sections. Values shown are mean ± SEM (n = 8/mouse; ***P < 0.001, vs. normal, #P < 0.05, vs. 2% MXD).

Analysis of β-catenin and Wnt3a expression in the skin of C57BL/6J mice
To evaluate the signalling mechanism underlying the induction of anagen phase in the HPE and HPE + MXD groups, we performed immunohistochemistry and immunofluorescence using antibodies specific to β-catenin and Wnt3a. Animals treated with HPE exhibited anagen induction, as evidenced by positive staining for β-catenin (Fig. 6a,b; upper panel) and Wnt3a (Fig. 6a,b; lower panel).
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Figure 6.
Representative sections of skin tissues of C57BL/6J mice stained for \( \beta \)-catenin and Wnt3a. (a) Dorsal skin biopsies taken after 21 days and immunohistochemically stained, with diaminobenzidine (DAB) as chromogen (red arrow indicates strong expression) with haematoxylin counterstain. (b) Immunofluorescence staining for \( \beta \)-catenin and Wnt3a (red, \( \beta \)-catenin; green, Wnt3a), with nuclei counterstained with DAPI (blue), \( n = 8 \) mice per group. Original magnification: \( \times 200 \).

Immunofluorescence revealed strong deposition of \( \beta \)-catenin and Wnt3a around hair follicles. In addition, the HPE + MXD group had markedly increased \( \beta \)-catenin and Wnt3a expression levels compared with the group treated with HPE only. In particular, anti- \( \beta \)-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 suggest that HPE activates certain functions in the anagen phase, and that HPE activates hair follicle development through the Wnt/ \( \beta \)-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 with hair loss and alopecia has increased dramatically in recent years.[17] There are various causes, including ageing, hormone imbalance, stress and nutritional deficiency, all of which can result in hair loss in both men and women.[18, 19] Two drugs are currently licensed for the treatment of male androgenic alopecia: oral finasteride[20] and topical MXD solution.[21] However, the efficacies of these two medications are limited and transient, owing to their 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] HPEs) a potent hair growth modulator, has been investigated as a promising candidate, using in vitro and in vivo studies. HPEs has been approved and is now widely used for tiredness alleviation, skin-whitening and antiageing applications.[6] Interestingly, a previous report showed that cow placenta extract was able to 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 can stimulate hair growth in animal models.[24] HPE contains 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 may assist in the development of new alternative medicines for the treatment of hair loss-related disorders, especially androgenic alopecia.

Cell viability was significantly and dose-dependently increased by a combination treatment of HPE + 0.5 μmol/L MXD compared with 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 colour was evaluated in order to determine changes in hair growth. Beginning on day 8 after depilation, the skin colour of the centre back region of mice in the HPE + MXD group was darker than that of the mice in the 2% MXD group. Likewise, the HPE + 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 + 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. Histological 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, indicating that HPE may stimulate early embryonic developmental processes and play a significant role in hair follicle development.

Wnt/β-catenin signalling promotes the development of new hair follicles, and is required for initiation of hair morphogenesis.[25-27] Within established hair follicles, Wnt cascade signalling plays a key role in the activation of bulge stem cells in order to stimulate hair formation, and this signalling is relayed by β-catenin and Lef1.[28] To explore the molecular mechanisms by which Wnt/β-catenin signalling 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 + MXD group had markedly increased expression of β-catenin and Wnt3a compared with the HPE-only group. These results indicate that the effects of HPE are 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 + MXD promotes hair follicle elongation in humans, thus extending the anagen phase. This suggests that HPE could be a potential treatment for 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 DPCs and promotes a 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.
Conclusion
In conclusion, the results of this study demonstrate that HPE has hair growth-promoting activity, probably through mechanisms acting via the Wnt/β-catenin pathway, thereby improving hair growth in a C57BL/6J mouse model. Our findings may assist in the development of a potential candidate combination therapy for hair loss. Additional experiments will be required to validate the results presented in this study.

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What’s already known about this topic?
There are currently two drugs that have been licensed for the treatment of male androgenetic alopecia, namely, oral finasteride and topical MXD solution, which are effective for hair regrowth. However, more effective alternative treatments are needed.

What does this study add?
We assessed the hair growth-promoting activity in mice treated with HPE or HPE in combination with MXD, and also investigated the underlying mechanism of action.
The combination of HPE and MXD promoted hair growth both in vitro and in vivo.