Effects of Neem (*Melia azadirachta* L.) Leaf Extract on Human Dermal Papilla Cells

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Abstract
In recent years, the potential of Neem (*Melia azadirachta* L) as an anti-aging supplement has been investigated in the health food industry. It has been traditionally used in Indian Ayurvedic Medicine (traditional system of medicine practiced in India) and also has been studied as an anti-cancer as well as anti-microbial agent. This study aimed to determine the effect of Neem leaf extract (NLE) on hair growth. To evaluate the anti-oxidant potential of NLE, we measured free radical scavenging activity of NLE through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, wherein NLE displayed significant DPPH-free radical scavenging activity in a dose-dependent manner. Treatment of human hair follicle dermal papilla cells (hHFDPCs) with NLE upregulated insulin-like growth factor-1 (IGF-1), which promotes hair growth. Furthermore, NLE down regulated hair-growth inhibitors, such as transforming growth factor-beta-1(TGF-beta-1), interleukin-1-alpha (IL-1-alpha), IL-1-beta, IL-6, and inducible nitric oxide synthase (iNOS). In addition, treatment with NLE down regulated the androgen receptor (AR) and dihydrotestosterone (DHT) enzyme 5-alpha-reductase in hHFDPCs. The results of the present study suggest that NLE promotes hair growth and inhibits hair loss by regulating the expression of hair growth factors and hair growth inhibitors.

Keywords: Hair cycle, hHFDPC (human hair follicle dermal papilla cells), NLE (Neem leaf extract)
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Introduction

Neem, a member of the Meliaceae family, is an evergreen tree grown in tropical regions and has traditionally been used as a form of Ayurvedic medicine. The potential of anti-inflammatory activity in NLE has been reported through previous studies and in this study, we focused on the effects of NLE on hair loss.

The hair follicle undergoes a cycle of growth (anagen), regression (catagen), and rest (telogen). It consists of epithelial and mesenchymal components, such as the outer root sheath (ORS), the inner root sheath (IRS), the matrix, and the connective tissue sheath (CTS). The mesenchymal portions also include dermal papilla (DP). The DP cells play a crucial role in development and cycling of the hair follicle through signaling with keratinocytesin the hair matrix (Dastan etal., 2016). During the anagen phase, DP cells express IGF-1, which promotes hair growth by regulating cellular proliferation and migration (Woo et al., 2017). However, while transitioning from the anagen to the catagen phase, the hair matrix cells stop proliferating and the catagen-inducing factors promote apoptosis of these cells (Dastan etal., 2016).

In recent years, many individuals have experienced hair loss caused by abnormal hair cycling, which, for example, includes shortening of the anagen phase, resulting in miniaturization of the hair follicles. There are many potential causes for hair loss, of which, increased sensitivity of the hair follicles to DHT is one (Woo et al., 2017). Shin et al. (2013) reported that DHT induced TGF-beta-1 expression, which inhibits cell growth in the hair follicle, andTGF-beta-1 expression induced by androgens was mediated by reactive oxygen species (ROS). Inflammatory cytokines, such as IL-1-alpha, IL-1-beta, IL-6, and iNOS are also known as inhibitors of hair growth.

In this study, we aimed to investigate the effect of NLE on the anagen phase and the transition from the anagen to the catagen phase, which promote hair growth.

Materials and Methods

1. Preparation of NLE

Neem (Melia azadirachta L) used in this study were cultivated in our laboratory (Institute of Traditional plants in Sri Lanka). The heat-treated these leaves were extracted with 1,3-butylene glycol.

2. Cell culture

Normal human hair follicle dermal papilla cells, hHFDPCs were provided by cell applications, INC.The cells were cultured in PCGM (TOYOBO) with 1% fetal calf serum (FCS), 0.5% insulin-transferrin-triiodothyronine mixture (ITT), 1% bovine pituitary extract (BPE) and 0.5% cyproterone acetate (Cyp). The cells were subjected to FCS, ITT, BPE and Cyp starvation for 16 hours with PCGM before all experiments.

3. RNA isolation and real-time PCR

Total RNA was extracted from hHFDPCs by using the TRI Reagent (Merck). It was used as a template for subsequent cDNA synthesis with oligodT primers by using the Prime Script RT reagent Kit (Takara Bio Inc.). mRNA levels were quantified by using a LightCycler 96 system (Roche) and SYBR Premix Ex Taq II (Takara Bio Inc.). The data were analyzed by using the delta cycle threshold method. The data were calculated based on the Cq values, and the expression of each gene was normalized to GAPDH. All values are reported as means ± standard error as previously described (Maeda-Sano et al., 2014)

4. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (assay for free radical scavenging)

The free radical scavenging capacity of plants extracts were analyzed by using DPPH . Plants extracts were diluted with 0.1M acetic acid buffer (pH 5.5) at various concentrations (0.01–1.0 % (volume/volume)). Ascorbic acid (1-10 µg/mL) were used as a positive control. A volume of 40µL of samples and 60 µL of ethanol (with or without 0.1mM DPPH) were mixed in 96-well plate at room temperature for 30 minutes, and the absorbance at 517 nm (A517) was measured. The DPPH scavenging effect was calculated as follows: Scavenging effect (%) = 100-(A-Ab) / (A0-A0b) ×100, where A0:A517 of DPPH without sample, A0b:A517 without sample and DPPH, A:A517 of sample and DPPH, and Ab:A517 of sample without DPPH.

Results and Discussion

At first, we determined free radical scavenging activity of NLE through the DPPH assay, and found that NLE displayed significant DPPH free radical scavenging activity in a dose-dependent manner. It is known that Reactive Oxygen Species (ROS) is one of the causes of inflammation; therefore, antioxidant activity could prevent inflammation. Furthermore, using NB1RGB human fibroblast cells, we previously reported that treatment with 0.01% NLE down regulated IL-1alphamRNA in approximately 50% of cases.IL-1alpha is a major inflammatory cytokine. These results suggest that NLE has anti-inflammatory activity. Hair loss can be cause by various factors; inflammation in the hair follicle is one of them. Therefore, we investigated the effect of NLE on human follicle dermal papilla cells (hHFDPCs).

We initially focused on the anagen phase of the hair follicle. To determine whether NLE affects IGF-1 expression, we first examined IGF-1 mRNA expression in hHFDPCs for 24 h by through quantitative real-time polymerase chain reaction (qRT-PCR). In hHFDPCs, treatment with 0.03% NLE increased IGF-1 mRNA expression. IGF-1 has been reported to promote hair growth by regulating cellular proliferation and migration. The present result suggests that NLE promotes hair growth by accelerating the expression of hair growth factor.

Furthermore, we focused on the transition from the anagen to the catagen phase. Here, the hair matrix cells stop proliferating and the catagen-inducing factors promote apoptosis of these cells. Hair loss caused by abnormal hair cycling, such as a shortened the anagen phase, results in miniaturization of the hair follicle. Various factors are reportedly involved in the transition from the anagen to the catagen phase; hence, we performed qRT-PCR analysis to determine whether NLE affects the expression of these factors in hHFDPCs. In hHFDPCs, treatment with 0.03% NLE down regulated 5-alpha-reductase, AR, and TGF-beta1 mRNA, with maximum suppression between 2 and 8 hours. DHT is synthesized from testosterone by the activity of 5-alpha-reductase, and the binding of DHT to AR in DP cells induces TGF-beta-1 expression, which is known to inhibit epithelial cell growth in the hair follicle. Foitzik *et al.* (2000) reported that TGF-beta-1 was associated with regulation of the catagen induction, possibly via inhibition of the hair follicle cells proliferation and induction of apoptosis. These results suggest that NLE prevents DP cells from undergoing apoptosis through inhibition of several factors involved in the transition from the anagen to the catagen phase. Furthermore, treatment with 0.03% NLE suppressed IL-1-alpha, IL-1-beta, IL-6, and iNOS mRNA expression with maximum suppression between 4 and 8 h. IL-1-alpha, IL-1-beta, IL-6, and iNOS are also known as inhibitors of hair growth. These results suggest that NLE promotes hair growth by down regulating inflammatory cytokines.

Conclusion

In conclusion, our findings suggest that (1) NLE promotes hair growth by accelerating the expression of hair growth factors and by suppressing the expression of inflammatory cytokines, and (2) NLE prevents DP cells from undergoing apoptosis through the inhibition of several factors involved in the transition from the anagen to the catagen phase. These results suggest that NLE has potential applications as a hair-growth–promoting agent.

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