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LETTER TO THE EDITOR

TOPICALLY APPLIED NICOTINAMIDE INHIBITS HUMAN HAIR FOLLICLE GROWTH *EX VIVO*

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Short title:

Nicotinamide inhibits human hair follicle growth

To the Editor,

The effective inhibition of unwanted human hair growth by safe and well-tolerated topical agents remains a major drawback of clinical and cosmetic dermatological treatments (Somani and Turvy 2014). One key challenge is to promote hair follicle (HF) regression (catagen) with negligible skin toxicity or irritation (S1). Pilot evidence suggests that nicotinamide, an amide form of vitamin B3 (Forbat et al. 2017), may be capable of doing this. That nicotinamide is used as a cosmetic and a dermatotherapeutic agent (Chen et al. 2016; Forbat et al. 2017; Niren 2006) (Supplementary Text ST1a) (Niren 2006; Walocko et al. 2017; S2) makes it an attractive candidate for hair growth inhibition. Therefore, we have investigated the hypothesis that nicotinamide may be an effective hair growth-inhibitor.

Micro-dissected human scalp HFs were obtained with institutional approval and written informed patient consent (Langan et al. 2015). These were cultured in the presence of 200 µM or 10 mM (Supplementary text ST1b) nicotinamide for 6 days, and key human hair biology read-out parameters were assessed (Kloepper et al. 2010; Ramot et al. 2014).

HFs treated with nicotinamide (10 mM) showed significantly decreased hair shaft production (Figure 1a) and entered catagen more rapidly than vehicle control HFs (Figure 1b, example staging Figure S1). This was confirmed by double-immunohistomorphometry (Ki-67/TUNEL), which showed significantly decreased hair matrix keratinocyte proliferation (Ki-67) below Auber's line (AL), increased apoptosis (TUNEL) within the hair matrix in anagen-phase HFs, showing a hair cycle independent effect of nicotinamide, (Figure 1c, d), with no effect on HF melanin content (Figure S2, Supplementary text ST1).

As nicotinamide added to culture medium imitates "systemic" application, we next asked if topical application to organ cultured human scalp skin (Supplementary Text ST2) (Lu et al. 2007) also inhibited human hair growth. Results showed that HFs treated with topical

nicotinamide (10 mM) entered and progressed through catagen faster than vehicle treated skin (day 3 and 6) (Figure 1e, Figure S1) with a corresponding, significant decrease in proliferation (Ki-67) (Figure 1f, g), mirroring HF organ culture experiments.

To exclude potential leaching of topical formulations into the medium we refined this assay by suspending the skin within an air-permeable membrane (see Supplementary Text ST2). In addition, we tested a modified topical nicotinamide formulation (i.e. 1% , matching 10 mM nicotinamide [Figs. 1a-g]) or 4% (common cosmetic concentrations (Navarrete-Solís et al. 2011, S2)), using a hydrosome-based vehicle, a clinical grade vehicle that promotes skin penetration (S3).

In this assay, the nicotinamide-hydrosome preparation (1% and 4%) significantly increased the number of catagen-like HFs (Figure 2a, Figure S1) (Oh et al. 2016). In line with this, the percentage of apoptotic (TUNEL+) HF matrix keratinocytes increased (Figure 2b, d) while the percentage of proliferating (Ki-67+) HF keratinocytes was significantly decreased (Figure 2c, d), with no effect on melanocyte proliferation (Figure S2). Moreover, topical nicotinamide (4%) also decreased the intrafollicular protein immunoreactivity for keratin 85, a surrogate marker for hair shaft production (S4) (Figure 2e, f), demonstrating hair growth inhibition was maintained using this vehicle.

As epilation devices and depilatory creams typically induce skin irritation and/or pruritus (S5), we examined potential adverse side-effects of nicotinamide by investigating mast cell (MC) granulation status, a sign of irritation, in the interfollicular dermis and HF connective

tissue sheath (CTS) using immunohistomorphometry of mast cell-tryptase (MCT). Contrary to previous reports showing nicotinamide reduced MC degranulation (Navarrete-Solís et al. 2011; Niren 2006)**,** 1% nicotinamide added to the HF medium significantly increased MC degranulation (Figure S3a, b) in the HF connective tissue sheath.

Comparing this to skin organ culture, no significant change in MC degranulation resulted from 1% nicotinamide-hydrosome applied topically, though a significant increase by 4% nicotinamide-hydrosome was detected (Figure 2g, h). Comparatively, nicotinamide-PEG6000 induced degranulation at a concentration of 1% (Figure 2i) suggesting a hydrosome vehicle may have some ability to reduce degranulation when used in combination with lower concentrations of nicotinamide (Discussion point Supplementary ST1f).

To further probe skin inflammation by topical nicotinamide, the number of CD68+ dermal macrophages was also investigated finding a significant decrease by 4% nicotinamide-hydrosome with a trend towards decreased numbers by 1% (Figure S4c, g). This suggests that nicotinamide does not induce lymphocytic inflammation but may induce itch and/or neurogenic inflammation. For this reason, a method for reducing MC degranulation whilst maintaining hair growth inhibition was examined.

For this purpose, palmitoylethanolamide (PEA, 30µM), an anti-pruritic endocannabinoid used in clinical dermatology, was applied to our nicotinamide/PEG6000 formulation (Supplementary text ST1b,c) and applied topically to *ex vivo* scalp sin to reduce MC degranulation (Parrella et al. 2016, S7).

The addition of PEA significantly reduced nicotinamide-induced dermal MC degranulation *ex vivo* (Figure 2i) without altering the hair growth inhibitory effect (Figure 2j). This supports the addition of PEA to future nicotinamide products to reduce MC degranulation-associated itch/inflammation while maintaining hair growth-inhibition (Supplementary text ST1d).

To examine the potential for topical nicotinamide-hydrosome (1% and 4%) formulations to cause additional epidermal side-effects which would render them unsuitable as a hair growth-inhibitory products (Cerchia and Lavecchia 2017), epidermal proliferation, apoptosis and pigmentation was assessed by quantitative immunohistomorphometry (Ki-67/TUNEL/Masson-Fontana). Neither 1% or 4% topical nicotinamide-hydrosome formulation significantly altered epidermal proliferation, apoptosis or pigmentation (Figure S4A,B, D-F) (supplementary text ST1e for discussion point).

Taken together these data present clear evidence that nicotinamide can effectively inhibit human hair growth and promote catagen in our clinically relevant models. That this occurred at commercially/clinically relevant concentrations without negatively affecting epidermal vitality or pigmentation makes nicotinamide an excellent cosmeceutical hair growth inhibitor for subsequent clinical testing. Data also suggest that hydrosomes provide a suitable vehicle and that PEA addition may reduce any potential skin irritation/itch that might be seen after repetitive application *in vivo*.

CONFLICT OF INTEREST

Data presented in Fig, 2A-H, S3 were supported by a grant to R.P. from RB, UK in the form of a research associate position for JAH.

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Figure Legends

Figure 1: Nicotinamide inhibits hair growth and induces catagen.

Human hair follicles (HFs) were cultured for 6 days with nicotinamide (200µM/10mM/vehicle) finding that (a) hair shaft elongation was decreased by 10mM nicotinamide with a (b) corresponding increase in catagen HFs (c,d), a significant decrease in Ki-67+ cells below Auber's line (AL) and an increase in TUNEL+ cells in the matrix compared to vehicle. When nicotinamide (10mM) was topically applied to human scalp skin, (e) a higher proportion of catagen HFs were detected (day 3) with more mid/late catagen at day 6 (f,g) and a matching decrease in Ki-67+ cells below AL compared to vehicle in anagen HFs. DP, dermal papilla; Data= mean; ± SEM; one-way ANOVA with Bonferroni's post-test. **P*<0.05, ***P*<0.01, and ****P*<0.001. Scale bar=50 µm.

Figure 2: Topical nicotinamide can reduce hair growth and prevent mast cell degranulation with the addition of PEA.

Using commercial formulations and a hydrosome vehicle showed that (a) both 1% and 4% nicotinamide induced catagen, (b) significantly increased TUNEL+ cells in the hair matrix and (c-d) significantly decreased Ki67+ below Auber's line. (e,f) As a surrogate marker for hair growth, Keratin 85 (K85) immunofluorescence was by nicotinamide (significant at 4%). (g) Notably topical nicotinamide (4%) did significantly induce mast cell degranulation in the interfollicular dermis which could be reduced by 30µM PEA (h, i) while maintaining hair growth inhibition (j). Data=mean± SEM; one-way ANOVA with bonferroni's post-test or student's unpaired t-test; w **P*<0.05, ***P*<0.01, and ****P*<0.001. Scale bar = 50 µm.