

A new strategy to modulate alopecia using a combination of two specific and unique ingredients

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Synopsis

Male pattern hair loss is a major cosmetic concern affecting both genders with a preference for men. Major causes of hair loss in genetically predisposed individuals include hormonal dysfunction, loss of extracellular matrix (ECM) proteins in the follicular bed, and localized microinflammation. Few options are yet available to correct the problem. For this purpose, a cosmetic active ingredient was developed by combining a *Trifolium pratense* flower extract and a biomimetic peptide and tested clinically for the prevention of hair loss. Thirty volunteers with receding hair were recruited for this randomized, placebo-controlled study. Clinical efficacy, following daily topical application of the mixture to the scalp, was checked using TrichoScan™ for the measurement of human hair. Within 4 months of application, anagen hair increased at an average by +13%, telogen hair density decreased by –29%, and the anagen/telogen (A/T) ratio increased by +46% over baseline in the treated group. Results strongly differed from those of the placebo group (anagen, –2%; telogen, +23%; A/T ratio, –33%). Investigation of the potential mechanisms involved in the positive effects of the test product on hair growth pointed at inhibition of 5- α -reductase activity, reduction of inflammatory reactions, and stimulation of ECM protein synthesis in the vicinity of the hair follicle.

INTRODUCTION

The presence of hair is a characteristic of mammals. Other species may have some kind of filamentous outgrowth but that certainly do not qualify as hair. Hair is a unique filamentous structure that extends from the dermis to the outside of the body, normally covering some areas entirely, such as the scalp, while sparing other zones, such as the palms and the soles. Human hair naturally comes in a variety of shapes, sizes, lengths, and colors, depending mainly on the specific genetic makeup of each individual. In mammals in general, hair serves diverse functions, such as providing protection, thermal regulation, camouflage, warning and mating signals, and a sense of touch (1). But for humans, it is

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even more than that. Hair is linked to our personality, appearance, sex, and social status; plays a role in seduction; and is instrumental to nonverbal communication. For all these reasons, the absence of hair, especially on the scalp, has a huge impact on one's life.

Each hair is composed of two distinct structures: the dynamic hair follicle located in the dermis and the hair shaft, a hard keratinized part that extends above the skin surface. The hair follicle is made of dermal and epidermal compartments closely interacting in the regulation of hair growth. The central structure of the hair follicle is the dermal papilla rich in mesenchymal cells (2). The papilla is connected to the capillary bed in the dermis and is embedded in a hair matrix consisting of epidermal cells capable of dividing rapidly to give rise to hair. The hair shaft comprises three layers: a cuticle, a medulla, and a cortex. Thickness of the shaft totally depends on the size of the papilla; the bigger the papilla, the stronger the shaft (3).

Hair grows in cycles delimited by three distinct phases: anagen, catagen, and telogen (Figure 1). Anagen is the growth phase during which new materials are deposited in the hair shaft by rapidly dividing follicular cells. Anagen scalp hair grows by 1 cm per month for a period of 2–6 years. The duration of the anagen period dictates the maximal length of hair and is genetically determined. Catagen is a transition phase, lasting for about 2–3 weeks, marked by a stop of hair growth. During this phase, the hair follicle involutes, becomes attached to the hair shaft and keratinizes forming a club hair that is pushed upward toward the scalp, as the dermal papilla breaks away. Telogen is the resting phase. The hair follicle regresses, becomes fully keratinized, and can easily be pulled out. The telogen phase lasts around 3 months for scalp hair. Following shedding, the next hair can start growing as the papilla and the follicle join again. An adult healthy scalp normally bears 70–85% hair in the anagen phase and 10–15% in the telogen phase, the rest being in the catagen phase (4). Male pattern alopecia is generally associated with a shortening of the anagen phase and premature entry into the catagen phase (5).

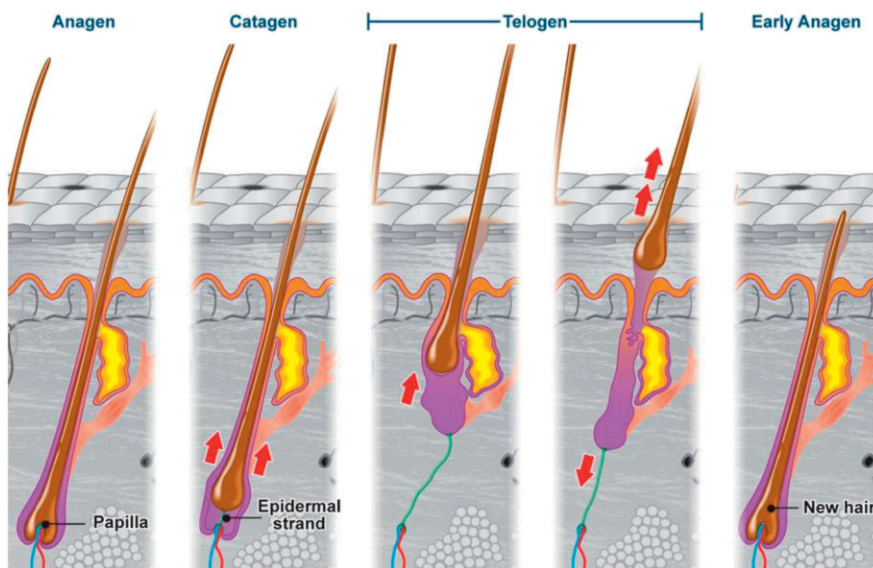


Figure 1. Normal hair physiology: hair cycles through anagen (growth phase), catagen (transition phase), and telogen (resting and falling phase) before reentering early anagen to initiate the growth of a new hair.

Up to a hundred hair are shed every day (6). Over that number, pathological hair loss (alopecia) is most likely to occur. Male pattern hair loss (androgenic alopecia) is the most common type of baldness, affecting roughly 50% of Caucasian men by the age of 50 years and 13% of Caucasian women before menopause increasing to 75% by the age of 65 years in women (5). Asians and African are less affected than Caucasians and the incidence is lowest in Native Americans and Eskimos (6). Patterns of hair loss may vary among genders. Indeed in men, the crown and temples are more likely to be first affected, a pattern that eventually progresses to baldness, whereas in women hair loss is generally rather diffuse (7). In most cases, hair thinning appears to precede hair loss (8).

The causes of male pattern hair loss are still a matter of debate, but genetic predisposition, hormonal dysfunction, loss of extracellular matrix (ECM) proteins in the follicular bed, and localized microinflammation are recognized as major triggers (Figure 2).

From a hormonal point of view, androgens are known to be important regulators of hair growth and the enzyme 5- α -reductase is pivotal to their effect. In the scalp, testosterone is metabolized to the stronger androgenic signal 5- α -dihydrotestosterone (DHT) by 5- α -reductase. Besides its action on androgen receptors in the follicle, DHT also stimulates the synthesis of transforming growth factor (TGF)- β in dermal papilla cells. TGF- β signaling is associated with inhibition of keratinocyte growth and induction of cell apoptosis (9). Pathological expression of TGF- β is a source of inflammation and fibrotic matrix deposition (10). In hair physiology, TGF- β is a catagen inducer that also prevents reentry from telogen to anagen, thus suppressing hair growth (11). Higher 5- α -reductase activity, resulting in high levels of DHT and dysregulated TGF- β signaling, is found in bald scalp (12).

Cell-matrix interactions are also key regulatory steps in hair cycling (13). The ECM is in constant remodeling during the different phases of hair growth. Maintenance of the ECM composition is mainly assumed by the dermal papilla fibroblasts, but proper exchange with hair matrix keratinocytes is mandatory for this function. These exchanges take place at the basement membrane zone (BMZ) located at the epithelial-mesenchymal interface of the hair follicle. Matrix proteins found at this interface serve as anchors to maintain

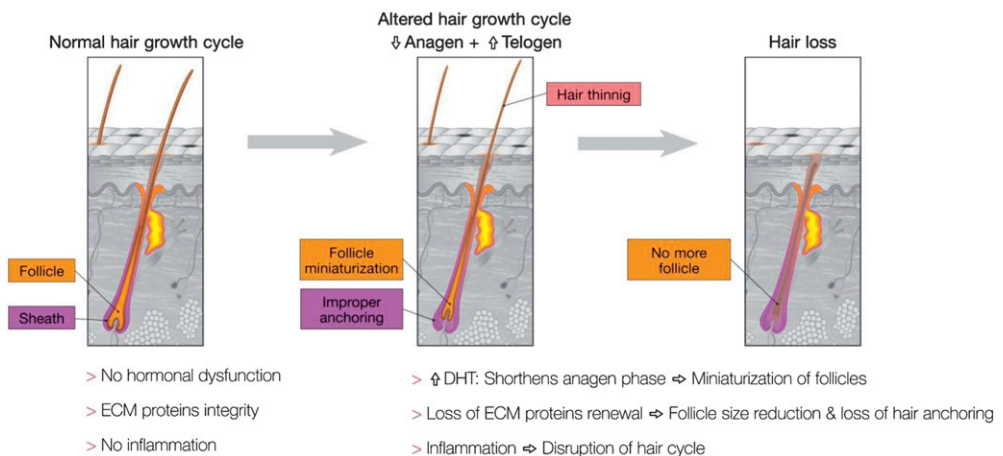


Figure 2. Pathological mechanisms involved in receding hair: combined effects of hormones, inflammation, and ECM dysfunction alter the hair growth cycle and lead to hair loss.

epithelial–mesenchymal contact and stabilize the BMZ. These include laminins, some integrins, and collagen VII (14,15). They have a crucial role in the maintenance of the hair follicle and the control of its volume.

Microinflammation is suspected to be a precipitating factor in male pattern alopecia (16), but the concept has yet to be integrated into treatment strategies. Exposure to irritants, pollution, and UV radiation has the potential to turn keratinocytes into mediators of inflammation (17). Under stress conditions, keratinocytes react by increasing their production of interleukin (IL)-1 α , a pro-inflammatory cytokine. The latter acts on fibroblasts to stimulate their production of IL-8, a cytokine involved in the recruitment of neutrophils. Both IL-1 α and IL-8 are inducible at the dermal papilla and were found in plucked hair samples of subjects with male pattern alopecia (18) suggesting their participation in the pathology (19). Cytokine-driven persistent inflammation also activates matrix metalloproteinases involved in tissue remodeling and perifollicular fibrosis (20).

Limited treatments are currently available for male pattern alopecia. The most popular are the following: minoxidil (Rogain[®] McNeil-PPC, Johnson & Johnson, New Brunswick, New Jersey, USA.), an over-the-counter vasodilator that is believed to optimize blood supply to the dermal papilla (5); finasteride (Propecia[®] Merck, Whitehouse Station, New Jersey), a drug that acts by inhibiting the enzyme that converts testosterone to DHT (5); and diaminopyrimidine oxide (Aminexil[®], l'Oréal, Paris, France), a patented compound that prevents perifollicular fibrosis. Each of these treatments targets one aspect of hair loss and offers a certain level of efficacy for those who respond. However, for improved results, it may be desirable to simultaneously target several aspects of the problem. The new cosmetic active ingredient (a mixture of clover extract and acetyl tetrapeptide-3), described in this article, represents a new, more integrative approach to hair loss.

MATERIAL AND METHODS

TEST MATERIAL

The test material consisted of a mixture of *Trifolium pratense* (clover) flower extract (total isoflavone $\geq 98\%$ and biochanin A $\geq 12\%$, determined by high-performance liquid chromatography (HPLC)) and acetyl tetrapeptide-3 (pure peptide obtained by solid phase peptide synthesis, purity $\geq 90\%$ determined by HPLC). The clover extract fraction is standardized using biochanin A, a phytoestrogen flavonoid with documented health-promoting activities (20). The tetrapeptide is a biomimetic derived from a signal peptide found in matrix proteins, such as collagen and fibrin, and also in HGF, which is a growth factor first isolated from human plasma (21). The peptide is normally liberated by proteolysis in the course of tissue damage. Its release and activation stimulates tissue remodeling following the initial phase of wound healing.

The components of the mixture were tested either together or alone, according to their expected roles in hair care, as could be deduced from the existing literature (20,22). An effect of the biochanin A component was documented on 5- α -reductase activity, while acetyl tetrapeptide-3 was investigated for its influence on ECM components, including collagens III and VII, and laminins. The mixture of both components was tested for anti-inflammatory activity before being clinically tested in humans to evidence efficacy in reducing hair loss.

5- α -REDUCTASE ACTIVITY

The enzyme 5- α -reductase catalyzes the conversion of testosterone to DHT. The effects of biochanin A on 5- α -reductase activity were studied in intact cells expressing type 1 or type 2 isoforms of the enzyme and compared to that of epigallocatechin-3-gallate (EGCG) from green tea. EGCG is a known in vitro inhibitor of 5- α -reductase (23). In this assay, radio-labeled testosterone served as substrate.

The activity of biochanin A on 5- α -reductase activity was shown by Hiipakka *et al.* (24). Briefly, 5- α -reductase-expressing cells were plated at 50,000 per well in a 24-well plate in specific medium for 18 h at 37°C. The medium was then changed to 0.5 ml of serum-free medium and 5 μ l of biochanin A (100 μ M) or EGCG (100 μ M) was added and kept for 1 h at 37°C before the addition of 14 C-testosterone, at a final concentration of 1.5 μ M. Cells were then incubated for an additional 3 h and radioactive steroids were extracted with ethyl acetate. The amounts of labeled testosterone and DHT in extracts were next determined by thin layer chromatography, as a measure of 5- α -reductase activity (for more details, see Ref. 24).

IMMUNOFLUORESCENT LABELING OF COLLAGEN III AND LAMININS

The effect of acetyl tetrapeptide-3 on the expression of different ECM proteins (collagen III and laminins) was evaluated by selective immunofluorescence in comparison with untreated fibroblasts. For this experiment, 3×10^4 human fibroblasts (MRC5 from ATCC CCL) were incubated in Dulbecco's modified Eagle's medium (DMEM) (Eurobio Laboratories, Courtaboeuf, France) containing 10% fetal calf serum (FCS) and supplemented with 1% penicillin/streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂ atmosphere to reach confluence. Cells were then incubated in the presence or absence of acetyl tetrapeptide-3 (10^{-7} M, equivalent to 0.05 ppm) for 3 days. These cells were rinsed with phosphate-buffered saline (PBS) and fixed on slides using methanol (for 10 min at -20°C) followed by acetone fixation (for 10 min, at 4°C). The slides were then dried at room temperature and rinsed with PBS at a pH of 7.6 for 10 min. The presence of collagen III and laminins in cells was detected by incubating the slides with specific antibodies diluted at 1/50° overnight at 4°C, that is, type III anti-collagen (rabbit, Rockland, Gilbertville, PA) and anti-laminin (rabbit, Sigma, St. Louis, MO), respectively. Detection of type III anti-collagen and anti-laminin antibodies was done using a goat anti-rabbit IgM + IgG rhodamine (TRITC) conjugate diluted at 1/100° (Southern Biotech, Birmingham, AL). The corresponding fluorescent signal was monitored using confocal microscopy (Axioplan and Zeiss LSM510 Oberkochen, Germany), allowing for semiquantitative evaluation.

IMMUNOHISTOLOGICAL LABELING OF COLLAGEN VII

The effect of acetyl tetrapeptide-3 on the expression of collagen VII, a major constituent of anchoring fibrils found in the middle part of the follicular BMZ and around the hair papilla was evaluated using immunohistological techniques. As the junction around the anagen hair follicle and its adjacent connective tissue is similar in terms of composition

and structure to the dermal–epidermal junction (DEJ) (25), the effect of acetyl tetrapeptide-3 on type VII collagen synthesis was evaluated on human skin explants.

Four human skin explants were obtained from abdominal part of patients undergoing plastic surgery (Caucasian women, 35–45 years old) and maintained in culture. On day 1, a corticoid cream (Diprosone® with 0.05% betamethasone) was applied at the surface of the skin explants to induce skin atrophy as seen in aging (26). Acetyl tetrapeptide-3 was then added to the culture media for 2 days at 10^{-5} M concentration (equivalent to 5 ppm). On day 3, the skin explants were prepared for specific collagen VII immunohistological labeling using Avidin-Biotin Complex ABC Peroxidase Kit (Vector Laboratories Burlingame, CA, USA) and revealed by AEC substrate (brown color).

Visual scoring of collagen VII expression ($n = 4$) was performed, using a scale ranging from 0 (negative) to 4 (maximum) defined as follows: 0, absence of the protein; 1, slight expression; 2, moderate expression; 3, normal expression; and 4, overexpression.

MODULATION OF IL-8 PRODUCTION

Low-grade chronic inflammation is increasingly seen as a contributing factor in male pattern alopecia (27). Under stress conditions, keratinocytes in the vicinity of the hair follicle may respond by releasing IL-1, a proinflammatory cytokine that commands the production of additional inflammatory agents such as IL-8 acting as chemoattractants for inflammatory cells. IL-1-induced IL-8 production in keratinocytes was used as a model to document the anti-inflammatory activity of red clover extract alone and in combination with acetyl tetrapeptide-3. Dexamethasone (DMS), a glucocorticoid with potent anti-inflammatory properties, served as a positive control for anti-inflammatory action.

Monolayers of cells derived from normal human dermal fibroblasts (NHDFs) (Life technologies, Saint Aubin, France) were cultured to confluence for 24 h in DMEM (Eurobio Laboratories) containing 10% FCS and supplemented with 200 mM L-glutamine and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂ atmosphere. Cells were then challenged by adding IL-1 α (0.0075 ng/ml from Eurobio Laboratories) to the culture media (without FCS) in the presence or absence of the test products (red clover extract alone or red clover extract + acetyl tetrapeptide-3) (0.5–1%) or DMS (1 μ M) for 24 h. At the end of this period, IL-1 α -induced IL-8 production was measured using a highly sensitive and specific enzyme immunoassay kit (human CXCL8/IL-8 DuoSet; R&D system Minneapolis, MN).

CLINICAL EFFICACY

Study population. Thirty healthy volunteers with active mild to moderate hair loss enrolled in the study. Patients were clinically evaluated and individual case histories were recorded to rule out possible pathologies such as iron deficiency anemia, thyroid-related conditions, or others that may influence hair growth. Patients were asked to use only “basic” shampoo and to avoid hair care treatment lotion according to the protocol. Hair count evaluation was done at the preselection step. As an inclusion criterion, less than 70% of all hair had to be in the anagen phase.

Study design. The study was designed as a randomized, placebo-controlled study. One half of the subjects (15 people) received the “active” lotion, whereas the other half was given a placebo lotion.

The active lotion composed of 5% of the test product formulated in a solution consisting of water (75%) and alcohol (20%); the placebo lotion composed of 80% water and 20% alcohol. The test product composed of a mixture of clover extract (titrated to reach 15 ppm biochanin A) and 300 ppm acetyl tetrapeptide-3.

The study lasted for 4 months (T4). Each evening, patients had to apply 20 drops of the test product or placebo lotion on balding areas and gently massage it into the whole scalp. For each week of the study, the patients received one plastic bag to collect all hair found on their pillow, comb, and clothes on a daily basis. The hair thus collected in a bag had to be returned to the laboratory for counts.

Evaluation procedures. Efficacy was objectively evaluated by instrumental measurements (digital trichogram with TrichoScan™). TrichoScan™ is a noninvasive method, combining standard epiluminescence microscopy with automatic digital image analysis, for the measurement of human hair (28,29). For determining total hair density, a mask was positioned on the volunteer head to delimitate a shaving area of 1.8 cm² on the zone to be studied. Three days later, as hair may not always contrast well enough with the scalp (due to the presence of gray or fair hair), hair was dyed and subsequently cleaned with alcohol. Images were then recorded with a camera for the purpose of hair count. Patients were asked not to wash their hair for 2 days before the TrichoScan™ examination. Following acquisition, the digital images were processed using software capable of analyzing anagen, telogen, and total hair density. The software was calibrated for an average anagen growth rate of 0.3 mm/day and no telogen growth.

According to the definition of the TrichoScan™ procedure, an anagen (A) hair is a hair that is detectable 3 days after complete hair shaving. Within this time, only anagen hair should grow significantly at a rate of approximately 0.3 mm/day. Nongrowing hair is by definition a terminal hair in the telogen (T) phase. The anagen/telogen (A/T) ratio is an indication of the percentage of active hair follicles.

↑ A/T ratio = activation of hair growth

↓ A/T ratio = loss of hair growth activity

RESULTS AND DISCUSSION

INHIBITION OF 5- α -REDUCTASE ACTIVITY

Biochanin A, a phytoestrogen that, like many other polyphenols including EGCG from green tea, has shown 5- α -reductase inhibitory activity in vitro (30). EGCG is also known to stimulate hair growth ex vivo (23,24).

In an intact cell assay, biochanin A proved to be a potent inhibitor of 5- α -reductase activity and was superior in that aspect to EGCG (Figure 3). In this assay, biochanin A (100 μ M) inhibited both type I and type II isoforms of 5- α -reductase by -64% and -93%, respectively, compared to those of -11% (type 1) and -5% (type 2) for EGCG. Both isomers are found in the scalp and there is strong evidence that type II contributes to male pattern

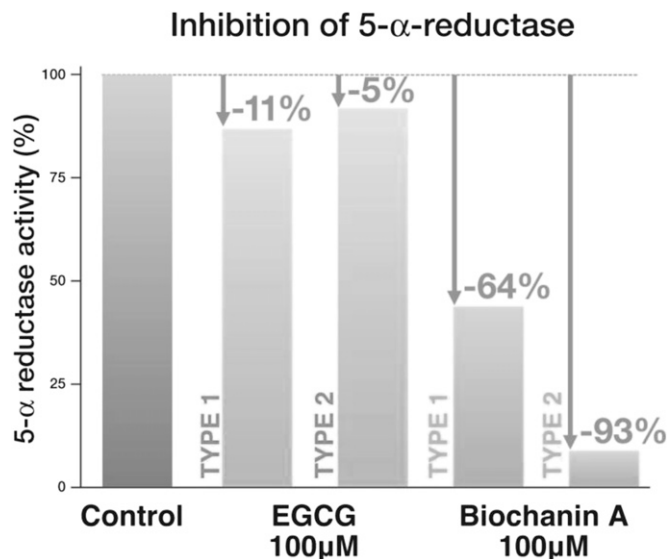


Figure 3. Inhibition of type I and type II isoforms of 5- α -reductase activity in intact cells by biochanin A and comparison to EGCG.

alopecia (31). This is supported by the fact that men genetically deficient in type II 5- α -reductase do not present hair loss (8). On the one hand, Finasteride, a selective inhibitor of type II 5- α -reductase, slows the progression of baldness in a majority of men (8,32) and also in some women (33). On the other hand, dutasteride, a dual inhibitor of type I and type II 5- α -reductase activities revealed to be even more potent than finasteride in improving hair growth in balding men (although not approved by the FDA for this indication), suggesting there might be an advantage in inhibiting both isozymes for this condition (31). These results suggest that extracts enriched in biochanin A, by inhibiting both type I and type II 5- α -reductase activity in the scalp, may find application in male pattern alopecia management.

STIMULATION OF ECM PROTEIN SYNTHESIS

The size of a hair follicle is thought to be determined by the volume of its dermal papilla, which depends on the number of cells and on the volume of the ECM (3). Regulation of the size of dermal papilla is a dynamic process involving hormonal influence and complex cell–matrix interactions. ECM proteins expressed in the hair follicle include collagens I, III, and VII, as well as laminins.

Collagens I and III. Type I and III collagens are the most abundant types of collagen in the skin. Not surprisingly, they are also found in the hair follicle where they have the particularity of presenting an unusual high ratio of collagen III/I. Collagen III is a fibrillar protein with elastic properties; it is tempting to speculate that these attributes might be of particular importance for hair follicle development and maintenance. Collagen I and III are produced in the human dermal papilla throughout the hair cycle (34) and are also major constituents of the connective tissue sheet of the hair follicle (4).

Laminins. Laminins are a family of large glycoproteins comprising more than 50 members that are the major constituents of basement membranes. They display a remarkable

repertoire of functions. Their interaction with integrin receptors at the surface of cells anchors skin cells to basement membranes and modulates cellular processes such as proliferation, apoptosis, differentiation, and motility (35). Laminin-511 (laminin-10) is the predominant laminin at the epithelial–mesenchymal interface of the hair follicle and is crucial for hair morphogenesis and anagen hair growth (36). Laminin-511 stimulates hair growth in vitro, and preventing its expression at the dermal papilla leads to hair regression and alopecia, a defect that can be prevented by application of the purified protein (37). Chemotherapy-induced alopecia has recently been associated with downregulation of laminin-511 expression, providing a new target for the prevention of this adverse effect (37). The effects of the matrix protein on hair follicle are mediated through the binding and activation of specific integrins at the surface of cells (38).

Collagen VII. Collagen VII is a long-chain collagen synthesized and secreted by keratinocytes and dermal fibroblasts. This matrix protein is a major component of anchoring fibrils that are specialized attachment complexes at epithelium–mesenchyme interface, in a number of tissues. In human skin, anchoring fibrils extend from the lower portion of the dermal–epidermal basement membrane to the underlying upper papillary dermis, forming U-like structures that entrap interstitial collagen fibers (39). In the hair follicle, collagen VII is found along the BMZ outside the hair follicle and at the dermal papilla junction inside the hair bulb. Collagen VII colocalized with integrins and laminins-511 at the epithelial–mesenchymal interface, suggesting a role in hair growth (14,15). The close interaction of collagen VII with laminins and interstitial collagen provides stability to the epithelial–mesenchymal interface.

Effects of acetyl tetrapeptide-3 on matrix proteins. The effect of acetyl tetrapeptide-3 (10^{-7} M) on the protein expression of type III collagen and total laminins in human fibroblasts (MRC5) was evaluated by selective immunofluorescence in comparison with untreated fibroblasts. Results showed a significant stimulation in the synthesis of both ECM proteins in the presence of the peptide (Figure 4); expression of type III collagen was increased by +65% while expression of laminins raised by +285% over untreated cells.

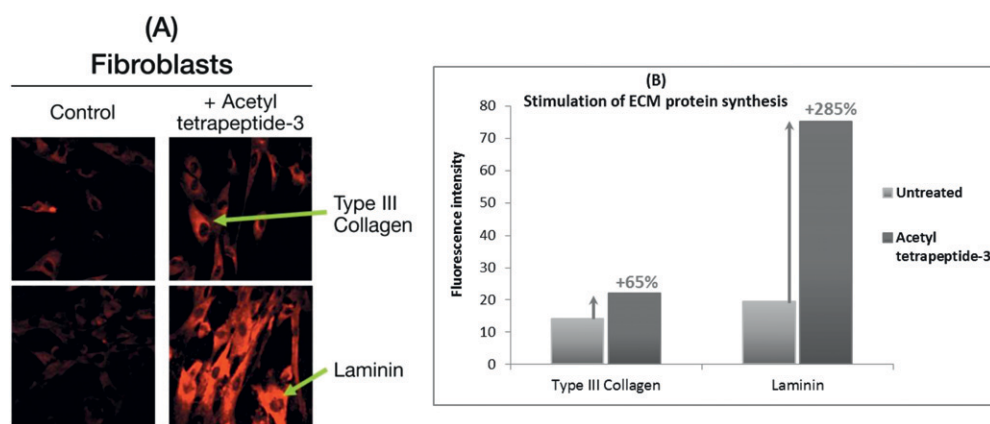


Figure 4. Effects of acetyl tetrapeptide-3 (10^{-7} M) on the protein expression levels of type III collagen and total laminins in human fibroblasts (MRC5); (A) immunofluorescence staining of type III collagen and laminins in control and acetyl tetrapeptide-3-treated cells; (B) histogram representation of results. Untreated cells served as control.

The potential effect of acetyl tetrapeptide-3 on protein expression levels of collagen VII at a mesenchymal–epithelial junction was documented using human skin explants. Skin atrophy was first induced through corticoid application in an effort to mimic the atrophic state of a deficient hair follicle. The explants were then treated with acetyl tetrapeptide-3, and collagen VII expression at the DEJ was revealed semiquantitatively by immunohistological staining. Microscopic observations of normal untreated skin revealed a strong labeling of collagen VII along the DEJ. Corticoid treatment resulted in a drastic and significant diminution (–70% on visual scoring) of collagen VII staining but baseline expression was completely restored in the presence of acetyl tetrapeptide-3 (Figure 5).

Given the importance of collagen III, collagen VII, and laminins for hair morphogenesis and growth, the positive impact that acetyl tetrapeptide-3 had on their expression in vitro and ex vivo supports the use of the peptide to help maintain an adequate ECM bed for optimized hair anchorage.

INHIBITION OF INFLAMMATORY SIGNALS

As mentioned in the Introduction section, microinflammation in the vicinity of the dermal papilla appears to precipitate or at least contribute to male pattern alopecia. To mimic inflammatory events that may prevail in this context, NHDFs were stimulated with IL-1 α in the presence or absence of red clover extract alone, or a mixture of red clover extract and acetyl tetrapeptide-3, at two different concentrations (0.5% and 1%). DMS, a glucocorticoid with anti-inflammatory properties, served as positive control for inhibition of IL-8 secretion.

As was expected, within 24 h of IL-1 α stimulation of NHDF cells, a massive release of IL-8 was observed in the culture media, an effect that was partially blocked by DMS (–17%). The production of IL-8 was also slightly inhibited in the presence of increasing concentrations (0.5% and 1%) of red clover extract alone (–11% and –20%, respectively). However, the mixture of red clover extract and acetyl tetrapeptide-3 was much more potent and showed a dose–effect relationship, inhibiting IL-8 release by –33% and –48%, at the lowest and the highest concentrations, respectively (Figure 6). From this

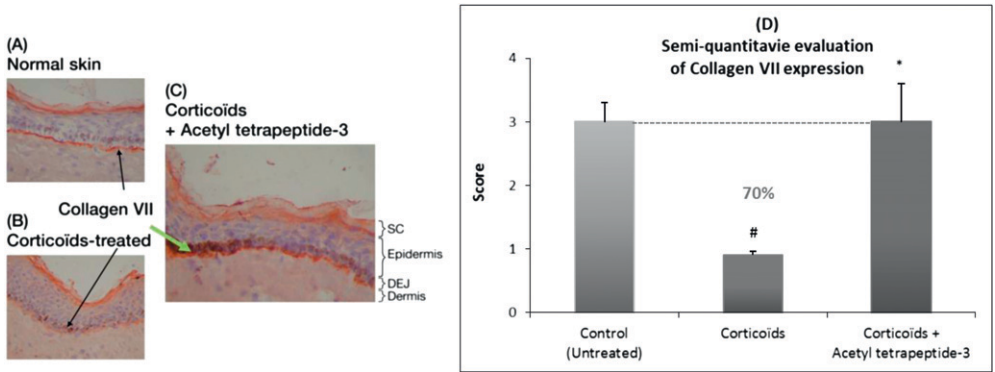


Figure 5. Effects of acetyl tetrapeptide-3 on protein expression levels of collagen VII at the DEJ, as measured by immunohistological labeling of human skin explants: (A) normal skin; (B) corticoid-treated (0.05%) skin; (C) corticoid-treated (0.05%) skin, cultured in the presence of acetyl tetrapeptide-3; and (D) visual scoring of collagen VII expression.

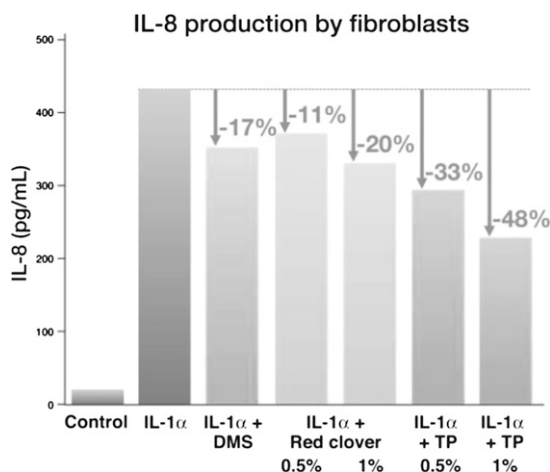


Figure 6. Effects of red clover extract (0.5% and 1%) and a mixture (TP) of acetyl tetrapeptide-3 and red clover extract (0.5% and 1%) on IL-1-induced IL-8 secretion from NHDFs. DMS at 1 μ M served as positive control for inhibition of IL-8 secretion.

experiment, it was concluded that the mixture of red clover extract and acetyl tetrapeptide-3 has the potential to modulate cutaneous inflammation in conditions similar to those found in the course of male pattern alopecia.

CLINICAL REDUCTION IN HAIR LOSS

Clinical efficacy of the mixture of red clover extract and acetyl tetrapeptide-3 (TP) in reducing hair loss was addressed using TrichoScan™. The technology combines epiluminescence microscopy with digital image analysis for the measurement of hair growth in vivo. The method is suitable for the determination of the density of hair (n/cm^2) in the anagen and telogen phases, the hair diameter (μm), the hair growth rate (mm/day), and the A/T ratio. For a more detailed description of the method, see the Material and Methods section.

Of the two groups of 15 participants originally enrolled, 15 placebo people and 14 treated people completed the 4-month study. The product or the placebo lotion was applied to the scalp at night, as described earlier. Overall tolerability of the topical formulations was excellent and no adverse events were reported. Results, which are expressed graphically in Figure 7, show that treatment with a mixture of red clover extract and acetyl tetrapeptide-3 statistically increased anagen hair density after 4 months of treatment ($p < 0.1$), whereas there is no statistic difference in the anagen hair density after treatment with the placebo lotion. Within 4 months, the number of hair in the anagen phase increased by an average of +13% in the treated group, whereas the number decreased by -2% in the placebo group. Treatment with the test product also induced a strong reduction in telogen hair density compared to the placebo group (Figure 7B). Average telogen hair density decreased by -29% over baseline in the treated group but increased by +23% in the placebo group, over the 4-month study. In accordance with these results, the A/T ratio increased significantly ($p \leq 0.05$) by +46% over baseline in the treated group, whereas it decreased significantly ($p \leq 0.05$) by -33% in the placebo group for the same period. The

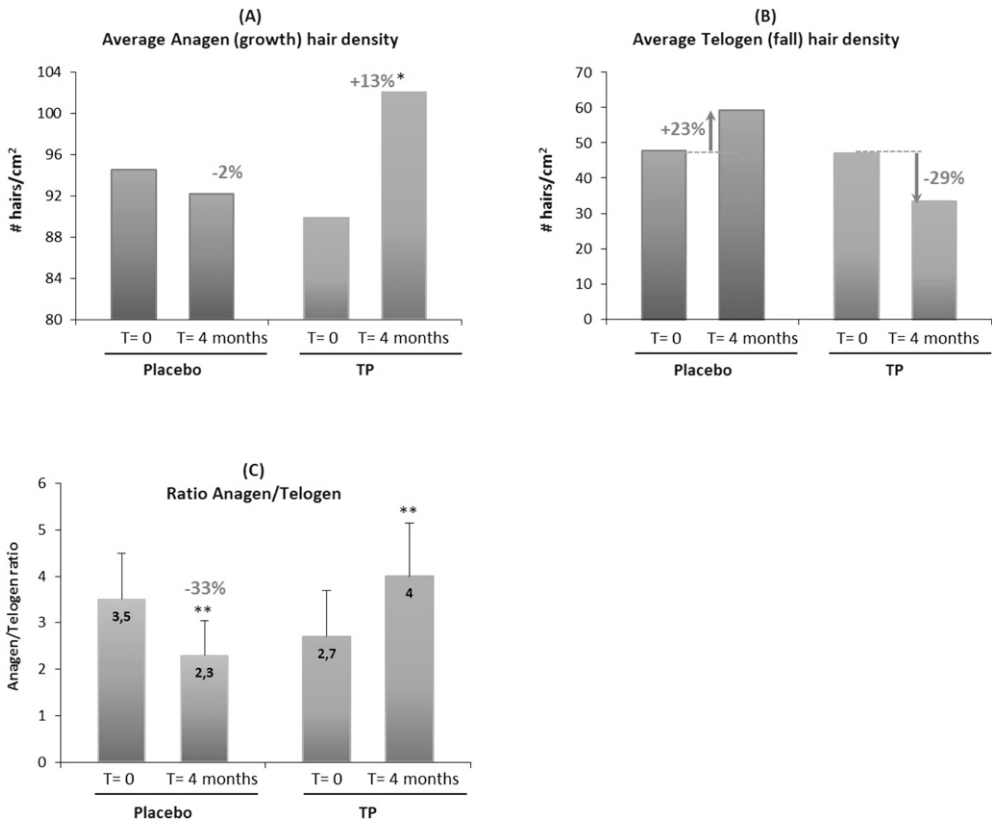


Figure 7. Effect of topical application of 20 drops of a 5% mixture of acetyl tetrapeptide-3 and red clover extract (TP) on hair cycle: (A) average anagen hair density at 4 months (T = 4) of treatment, compared to baseline (T = 0); (B) average telogen hair density at 4 months of treatment (T = 4), compared to baseline (T = 0); and (C) average ratio of hair in the anagen versus telogen phase of the hair cycle, at 4 months of treatment (T = 4), compared to baseline (T = 0).

A/T ratio being a comparison of the number of anagen to telogen hair follicles, the observed increase in A/T ratio attests that the test product is able to promote hair growth in people suffering from receding hair.

CONCLUSION

Taken together, these results support the efficacy of the test product in improving hair growth by rebalancing the A/T ratio and promoting healthy hair growth. The mixture combines the benefits of both biochanin A-enriched red clover extract and biomimetic acetyl tetrapeptide-3 to simultaneously address multiple factors involved in the progression of male pattern alopecia. The mixture acts on receding hair by limiting hormonal influence through inhibition of 5- α -reductase activity, preventing aggravation of hair loss due to microinflammation and supporting hair growth through stimulation of ECM protein synthesis. Clinical efficacy in supporting hair growth was successfully addressed in a 4-month randomized, placebo-controlled study involving volunteers presenting signs of male pattern alopecia.

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