ABSTRACT

Objectives: Plant extracts possessing specific constituents with anti-inflammatory, antimicrobial, antioxidant, or 5α-reductase inhibitory properties are known to provide benefits against androgenetic alopecia (AGA) in men. A solid shampoo was formulated, and it contained a mixture of six different plant extracts that possess these beneficial properties against AGA. The improvement in AGA and changes in steroid concentrations were assessed after 4 months of formulated shampoo use.

Methods: This study was conducted based on a randomized, placebo-controlled, and single-blind design. Hair-related variables and hair and saliva samples were collected bi-monthly in the treatment (n=48) and placebo (n=52) groups and at a single time point in the hairy controls (n=50).

Results: The formulated shampoo was more effective on AGA than the placebo based on the hair shaft thickness and hair density in the receding hairline. The baseline hair cortisol and dihydrotestosterone (DHT) concentrations were significantly higher in the treatment and placebo groups than in the hairy controls. After 4 months, the hair steroid concentrations in the treatment group were reduced to those observed in the hairy controls, although the main effect of time on hair steroid concentrations was negligible in the placebo group. Salivary cortisol and DHT levels during the post-awakening period were comparable among the groups or assessment time points.

Conclusion: The constituents of plant extracts included in the formulated shampoo would prevent hair loss, increase hair growth effects, and reduce hair cortisol and DHT concentrations without changes in the post-awakening salivary steroid levels in men with AGA.

Keywords: Androgenetic alopecia, Plant extracts, Local stress response system, Hair cortisol, Hair dihydrotestosterone.

INTRODUCTION

Androgenetic alopecia (AGA) is the most common type of hair loss in adult men, and it is characterized by the progressive loss of terminal hairs with a characteristic pattern distribution [1]. It is generally accepted that dihydrotestosterone (DHT) is the primary androgen associated with the development and progression of AGA in men [2], and finasteride, an inhibitor of 5α-reductase, is commonly used for the treatment of AGA [3]. The majority of dermatologists prescribed finasteride (1 mg/day) for more than one year to improve alopecia, and it slows hair loss and increases hair growth in men with AGA [4]. However, the beneficial effects of finasteride on AGA disappear after 12 months after the withdrawal of therapy [5], indicating that the continued use of finasteride is required to maintain the beneficial effects. Long-term use of finasteride can also induce adverse effects on sexual function (i.e., low libido, erectile dysfunction, and decreased ejaculation) and central nervous function and lead to depression and anxiety due to the concomitant inhibition of 5α-reductase that occurs in the central nervous system and peripheral organs [6].

The adverse effects of finasteride have led to an increasing interest in alternative remedies that can prevent and treat AGA in men. Plant-derived products, such as extracts and essential oils, have been traditionally used to treat alopecia, and recent human studies have observed that hair growth is promoted and hair loss is reduced in men with AGA after the topical use of plant extracts and essential oils [7,8]. Although none of the isolated chemical constituents of plants have been studied for their activity, the constituents of plant extracts and essential oils possessing anti-inflammatory, antioxidant, antimicrobial, or 5α-reductase inhibitory effects are known to have beneficial effects on alopecia [9]. A systematic review of cell culture and animal studies has suggested that the active constituents of plant extracts and essential oils may promote hair growth and inhibit hair loss through physiological changes in hair follicles and scalp skin, including the reduction of DHT formation by inhibition of 5α-reductase activity and modulation of hair growth stimulating and inhibiting factor expression, such as insulin-like growth factor-1, fibroblast growth factor, keratinocyte growth factor, and transforming growth factor β (TGF-β) [10].

Meanwhile, elevated DHT concentrations and DHT-related factors, such as 5α-reductase activity in scalp skin and hair follicles, are known to be associated with AGA development in men. For example, 5α-reductase activity in hair follicles collected from scalp skin is higher in men with AGA than in normal hairy men [11], and DHT concentrations are higher in biopsied scalp skin and hair samples collected from alopecic areas than unaffected hairy areas in the same subjects [12,13,14]. Another study line has shown that the topical use of finasteride provides positive outcomes of AGA without changes in circulating testosterone and DHT concentrations [15]. AGA improvements have also been observed after the topical use of anti-inflammatory chemical agents, such as roxithromycin [16], and antimicrobial and antifungal agents, such as piroctone olamine and triclosan [17], suggesting that topical treatment with agents possessing antimicrobial and anti-inflammatory properties mimics the beneficial effect of finasteride on AGA.
However, the physiological mechanism underlying the improvement of AGA after the topical use of plant products remains poorly understood. Based on the expression of mRNAs for 5α-reductase and other steroidogenic enzymes in hair follicles and sebaceous glands and the inhibitory effects of proinflammatory cytokines on hair growth, it has been postulated that cross-talk may occur between proinflammatory cytokines and steroidogenic activity in peripheral organs that disrupts the normal hair cycle [17]. Indeed, recent studies have confirmed that skin and hair follicles have their own proteins, enzymes, and self-regulatory feedback systems by which steroid hormones, including cortisol, are synthesized de novo in response to corticotropin-releasing hormone (CRH) stimulation and homeostasis-disrupting stimuli, such as ultraviolet radiation and proinflammatory cytokines, independent of central hypothalamus-pituitary-adrenal (HPA) axis function [18-20].

Given the presence of the stress response system and the variety of enzymes required for steroid metabolism in skin and hair follicles, the present study hypothesized that topical treatment with plant extracts possessing anti-inflammatory, antimicrobial, and 5α-reductase inhibitory properties might reduce the stress burden imposed on the local stress response system and improve AGA through a decrease in local steroidogenic activities, including DHT and cortisol formation. To examine this hypothesis, we formulated a solid shampoo that contains a mixture of plant extracts that possess these beneficial properties against AGA. The hair-related variables and steroid concentrations in the hair and saliva samples were determined in men with AGA after using the formulated shampoo.

METHODS

Study subjects

Men aged 20–65 with patterned hair loss and those with little or no hair loss were recruited from Gwangju Women’s University (GWU) through advertisements and posters. Subjects were excluded if they met any of the following criteria: (1) Diagnosis of any skin disorders of the scalp; (2) history of finasteride administration or current administration of finasteride; (3) topical minoxidil solution use, either currently or within the last month; (4) use of cosmetic hair dye, and (5) hair length <2 cm at the vertex region of the head. Subjects were initially screened in a telephone interview to exclude those with conditions known within the last month; (4) use of cosmetic hair dye, and (5) hair length <2 cm at the vertex region of the head. Subjects were initially screened in a telephone interview to exclude those with conditions known to influence central HPA axis function according to the prespecified exclusion criteria [21].

A text message was sent to each participant to notify them of their scheduled date. During their visit to the laboratory, their weight and height were measured, and each participant was asked to complete a questionnaire related to hair loss and other health conditions. Hair loss was assessed as a reliable index for central HPA axis function [31]. To determine the CAR, the participants provided their saliva samples in the morning, 2 hours after waking up, at 8 AM, and the cortisol awakening response (CAR) is used to assess the stress response system. In the placebo group, the participants used an unmedicated liquid cleansing shampoo over the same period. Participants in the treatment and placebo groups were not informed about the specific nature of the shampoo, and they were asked to report any adverse events during shampoo use, such as stinging, burning, and itching. Fifty men with little or no hair loss were also included as healthy controls.

Collection of hair samples and assessment of hair-related variables

After applying the exclusion criteria, the eligible men with AGA were randomly assigned into two groups (placebo group: n=52, treatment group: n=48) using a research randomizer (https://www.randomizer.org). Men with AGA in the treatment group used the formulated shampoo containing JU7505 over four consecutive months, and those in the placebo group used an unmedicated liquid cleansing shampoo over the same period. Participants in the treatment and placebo groups were not informed about the specific nature of the shampoo, and they were asked to report any adverse events during shampoo use, such as stinging, burning, and itching. Fifty men with little or no hair loss were also included as healthy controls.

Hair samples and hair-related variables were collected bimonthly for four months from men with AGA. The hair samples were collected from the receding hairline, a transition zone between the normal hair area and balding area (e.g., temples, vertex scalp, and/or mid-frontal area) in men with AGA before (t0) and at 2 (t1) and 4 (t2) months after using the provided solid shampoo. Hair samples were also collected at a single time point (t0) in the corresponding areas in the healthy controls. Approximately 70–100 mg of hair collected 2 cm from the scalp was obtained from the target areas using scissors, and the areas were photographed. An Aramco TS phototrichogram system equipped with magnification lenses (Aram HUVIS Co., Korea) was used to evaluate hair-related variables (hair shaft diameter and hair density). The hair-related variables at the target site were evaluated by three independent investigators 3 times, each in a double-blind fashion.

Collection of saliva samples

The concentrations of steroids in saliva are known to reflect those in the blood [30], and the cortisol awakening response (CAR) is used as a reliable index for central HPA axis function [31]. To determine the CAR and testosterone, DHT, epi-testosterone (Epi-T), and dehydroepiandrosterone (DHEA) levels during the post-wakening period, a set of saliva samples (immediately after waking and 30 and 60 min after awakening) was consecutively collected from men with AGA at each time point (t0) and at a single time point (t0) from healthy controls. Each participant was instructed on the procedures and precautions for saliva collection described in previous studies [32,33]. Each participant was also asked to submit their saliva samples at each scheduled visit for hair collection and collect their saliva samples on one of the working days, with a minimum volume of 1.5 ml of saliva provided at each time point.

Preparation of hair and saliva samples for steroid assay

Steroids were extracted directly from the finely cut hair following previously reported methods [34,35]. Briefly, approximately 50 mg of...
hair was cut into small pieces (2–3 mm in length) with small surgical scissors and then placed into a disposable glass scintillation vial to extract hair steroids. Then, 2 ml of methanol was added to each vial. Each tightly capped vial was incubated for 24 h at RT with gentle shaking using an orbital shaker (Jeio Tech, Korea). The next day, the tubes were briefly spun in a vortex, and 1.5 ml of supernatant was transferred into a clean tube and evaporated at RT in a centrifuge-type vacuum drier (Thermo Fisher Scientific, MA, USA). The surnps were resuspended in 1 ml of 0.1% gelatin containing 50 mmol/L phosphate-buffered saline (pH 7.2), and an aliquot of 500 μL was taken for the DHT assay. All samples were stored at −80°C until the assay was performed. The collected saliva samples were centrifuged (10,000 g for 15 min at 4°C) to remove debris. The supernatant was mixed with the same volume of GPBS, and then an aliquot of 500 μL was taken for DHT assay. The prepared saliva samples were stored at −80°C until the assay was performed.

Exclusion of saliva samples collected from non-adherent participants

Saliva samples that had insufficient volume and showed visible blood or sputum contamination were excluded. After exclusion, a cortisol assay was performed to evaluate whether the saliva samples were collected as described above. Typical CAR is defined as an increase in cortisol levels of at least 25 nmol/L above an individual’s baseline in healthy subjects [36]. However, the first sample collection after a delay of more than 10 min post waking is known as the leading cause of failure to capture a typical CAR in healthy subjects (that is, non-adherent subjects) [37]. We did not observe the typical CAR, and either a <2.5 nmol/L increase or the complete absence of a post-awakening increase was observed in some participants’ saliva samples. For example, at t0, the cortisol concentrations immediately on waking and at 30 and 60 min after waking in some of the men with AGA (n=31) were 20.9±2.2, 17.5±2.1, and 11.5±1.5 nmol/L (mean±SEM), respectively, while those in some of the healthy men (n=14) were 19.0±3.4, 13.4±1.7, and 9.6±1.3 nmol/L (mean±SEM), respectively. The subjects without a typical CAR were considered non-adherents, and data obtained from the non-adherents were excluded from further analyses. Finally, data were obtained from 36, 18, and 18 adherents in the treatment group at t0, t1, and t2, respectively; 26, 15, and 16 adherents in the placebo group at t0, t1, and t2, respectively; and 33 adherents in the hairy controls.

Steroid RIA

The cortisol, testosterone, DHT, Epi-T, and DHEA concentrations in the collected hair and saliva samples were determined using radioimmunoassay by modifying the method used in previous studies [32,33]. DHT assays were conducted after the selective inactivation of testosterone with an aqueous potassium permanganate solution [38]. The reference standards for the steroids were obtained from Sigma-Aldrich (MO, USA). Iodine-125-labeled cortisol, testosterone, DHT, Epi-T, and DHEA were prepared by modifying a previously described protocol for steroid radioiodination [39]. Cortisol antiserum was purchased from USBiological Life Sciences (MA, USA). Testosterone and DHT antiserum were purchased from Creative Diagnostics (NY, USA). As described by the suppliers, the cortisol antiserum cross-reacted with 11-deoxycortisol, prednisolone, corticosterone, cortisone, and 11-deoxycorticosterone, with cross-reaction levels of 0.9%, 5.6%, 0.6%, 0.6%, and <0.1%, respectively. Testosterone antiserum cross-reacted with 11β-hydroxy testosterone, 17α-methyltestosterone, DHT, estradiol, and progesterone, with cross-reaction levels of 3.3%, <0.1%, 0.8%, <0.1%, and <0.1%, respectively. DHT antiserum cross-reacted with testosterone, 5α-androsten-3β, 17β-diol, 5α-androstan-3α, 17β-diol, epitestosterone, and other steroids, with cross-reaction levels of 75%, 2.0%, 1.0%, 1.1%, and <0.1%, respectively. DHEA antiserum cross-reacted with DHEAS, androstosterone, androstenedione, 20-dihydropregesterone or 11-hydroxyprogesterone, with cross-reaction levels of <0.1%.

The standards, quality control materials, and samples were assayed in duplicate. The immunoassays of concentration (CVs) assessed from quality controls with mean cortisol concentrations of 3.6 and 10.9 nmol/L were 7.6% and 8.7%, respectively (n=18). The analytical sensitivity for cortisol was 0.4 nmol/L. The immunoassays of the quality controls with mean testosterone concentrations of 34.7 and 173.3 pmol/L were 8.9% and 7.6%, respectively (n=22). The analytical sensitivity for testosterone was 0.3 pmol/L. The immunoassays of the quality controls with mean DHT concentrations of 17.0 and 516.4 pmol/L were 9.6% and 8.7%, respectively (n=19). The analytical sensitivity for DHT was 0.3 pmol/L. Epi-T antiserum cross-reacted with testosterone, 5α-androsten-3β, β-dione, DHT, aldosterone, cortisol, and progesterone, with cross-reaction levels of <1%, <0.3%, <1%, 0.015%, and <0.1%, respectively. The immunoassays of the quality controls with mean Epi-T concentrations of 34.7 and 173.3 pmol/L were 9.4% and 8.3%, respectively (n=20). The analytical sensitivity for epi-testosterone was 0.3 pmol/L. The immunoassays of the quality controls with mean DHEA concentrations of 28.8 and 144.2 nmol/L were 8.7% and 8.0%, respectively (n=20). The analytical sensitivity for DHEA was 0.4 nmol/L.

Data analyses

Student’s t-test or Mann–Whitney’s U-test was applied according to the data distribution (Shapiro-Wilk test) to evaluate the differences between the two examined groups. The Chi-square test was used to analyze the differences in the categorical demographic data. The differences between three or more groups were determined with a parametric or nonparametric one-way ANOVA. The differences in the levels and patterns of cortisol, testosterone, DHT, Epi-T, and DHEA secretions during the post-awakening period were calculated as the area under the curve (AUC) with respect to the ground from the point immediately after awakening to 60 min after awakening (CARAUC, TaUC, DaUC, EpiTauc, or DaTauc, respectively). The relationships between the steroid concentrations were analyzed using Pearson’s correlation test. The results are expressed as the mean±SEM, and a p<0.05 was considered statistically significant. NCSS 11 statistical software (NCSS, UT, USA) was used for the data analysis.

RESULTS

Differences in demographics and self-reported variables between examined groups

Table 1 summarizes the demographic characteristics of each group. The mean age of the hairy controls was significantly lower than that of the treatment group (36.6±4.1 vs 40.0±6.9, p<0.001) and placebo groups (26.2±5.8 vs 40.0±6.9, p<0.001). There were no differences in the prevalence rates of the type of hair loss between the treatment and placebo groups (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001). Type M, MC/MO/CO, and O regressions were more prevalent in the treatment group (χ²=20.5, p<0.0001).
Table 1: Demographic characteristics

| Demographic variables                  | Treatment group (n=48) | Placebo group (n=52) | Hairy controls (n=50) |
|----------------------------------------|------------------------|----------------------|-----------------------|
| Age (year)                             | 49.0                   | 47.7                 | 41.24                 |
|                                         | (29–65)*               | (30–61)              | (22–63)               |
| Height (cm)                            | 172.1                  | 172.6                | 174.8                 |
|                                         | (160–192)              | (165–183)            | (161–186)             |
| Body weight (kg)                       | 73.5                   | 75.6                 | 79.3                  |
|                                         | (55–117)               | (55–92)              | (63–110)              |
| Smoking status                         |                        |                      |                       |
| Never                                  | 21.0%                  | 23.6%                | 35.0%                 |
| Quit                                   | 35.5%                  | 16.4%                | 15.0%                 |
| Current                                | 43.5%                  | 60.0%                | 50.0%                 |
| Quit period of stop                    | 1.66                   | 19.1                 | 9.64                  |
| smokingw (year)                        | (2–30)                 | (8–30)               | (5–20)                |
| Amount of smoking of quit-smoker       | 1.34                   | 13.9                 | 16.5                  |
| smoker (cigarettes/day)                | (2–30)                 | (10–20)              | (7–30)                |
| Smoking period of current-smoker       | 2.25                   | 23.9                 | 17.1                  |
| smoker (year)                          | (6–36)                 | (10–40)              | (3–40)                |
| Amount of smoking of current-smoker    | 1.74                   | 16.0                 | 16.4                  |
| current-smoker (cigarettes/day)        | (10–40)                | (2–40)               | (2–40)                |
| Drinking days                          | 1.7                    | 1.4                  | 1.7                   |
| (days/week)                            | (0–7)                  | (0–6)                | (0–7)                 |
| Amount of drinking (cups of bear/week) | 6.7                    | 5.7                  | 7.6                   |
| Family history of AGA                  |                        |                      |                       |
| Presence                               | 65.6%                  | 65.5%                | 33.0%                 |
| Absence                                | 34.4%                  | 34.5%                | 67.0%                 |
| Who is affected? (multiple marking)    |                        |                      |                       |
| Paternal side                          | 93.1%                  | 92.8%                | 90.0%                 |
| Maternal side                          | 6.9%                   | 14.3%                | 10.0%                 |
| Brother (or sister)                    | 24.1%                  | 32.1%                | 20.0%                 |
| Paternal side grandfather              | 17.4%                  | 14.3%                | 15.0%                 |
| Maternal side grandfather              | 3.4%                   | 2.5%                 | 2.5%                  |
| Duration of AGA                        | 10.6                   | 9.2                  | 13.0 (1–30)           |
| Type of AGA                            | O+MC type:             | O+MC type:           |                       |
|                                         | 36.7%                  | 36.3%                |                       |
|                                         | C                      | C                    |                       |
|                                         | type: 13.3%            | type: 7.3%           |                       |
|                                         | type: 6%               | type: 0%             |                       |
|                                         | 30.0%                  | 30.9%                |                       |
|                                         | M type: 20%            | M type: 12.7%        |                       |

*Data are presented as mean (range). AGA: androgenetic alopecia

Differences in hair-related variables between the examined groups

Fig. 1 presents the hair shaft thickness and hair density in the examined groups and shows differences in these hair-related variables between the examined groups at each different sampling time point. The hair shaft thickness and hair density obtained from the placebo and treatment groups at t0, t1, and t2 were significantly different from those obtained from the hairy controls (F_{1,55}=11.89, p<0.0001). The main effect of the time of sampling on the hair shaft thickness and hair density was significant in the treatment group (all t<1.3, p<0.05) (Fig. 1a and b). The main effect of the sampling time on the hair shaft thickness or hair density was negligible in the placebo group (all t>1.3, df=96, p>0.05) (Fig. 2a-e).

The time effects on the concentrations of hair cortisol, DHT, Epi-T, and DHEA at t0 among the examined groups were significantly different (F_{1,55}=9.8, p<0.001), and the post hoc tests revealed that hair cortisol and DHT concentrations were significantly higher in the treatment and placebo groups than in the hairy controls (p<0.001 for all) (Fig. 2a and c). However, the hair testosterone, Epi-T, and DHEA concentrations at t0 were comparable among the examined groups (F_{2,147}=0.4, p>0.05) (Fig. 2b, d, and e).

The time effects on the concentrations of hair steroids during the examined periods (t0, t1, and t2) in the placebo and treatment groups and the concentrations of hair steroids determined during the examined periods (t0, t1, and t2) in the examined groups at each different sampling time point. The hair shaft thickness and hair density obtained from the placebo and treatment groups at t0, t1, and t2 were significantly different from those obtained from the hairy controls (F_{1,55}=11.89, p<0.0001), and both hair-related variables were significantly greater at t2 than t0 (p<0.001 for all post hoc tests) (Fig. 1a and b). However, the main effect of the time of sampling on the hair shaft thickness or hair density was negligible in the placebo group (all F_{1,55}<1.82, p>0.05) (Fig. 1a and b). The hair shaft thickness measured at t2 in the treatment group was significantly lower than that measured at t0 in the hairy controls (p<0.05 by post hoc tests), although the hair density measured at t2 in the treatment group was comparable to that in the hairy controls (p>0.05 by post hoc tests) (Fig. 1a and b).

In the treatment group, the mean hair shaft thickness and hair density increased by 14.6% and 29.8% at t2, respectively, compared with those at t0.
The present study examined hair-related variables and steroid levels in hair and saliva samples of AGA in men after using a formulated shampoo containing plant extracts (JU7505) to examine the physiological effect of these plant extracts. The findings of the present study were twofold: (1) the hair shaft thickness and hair density increased along receding hairlines, and (2) the concentrations of hair steroids decreased while the levels and patterns of salivary steroids did not show significant changes during the post-awakening period in men with AGA after four months of formulated shampoo use.

We observed that the treatment group had an increased hair shaft thickness and hair density along receding hairlines after four months of formulated shampoo use, indicating the improvement of AGA, with reduced hair loss and increased hair growth, by the active constituents of JU7505. The results were similar to those of previous human studies that reported the improvement of alopecia in men with AGA after using a hair tonic containing crude extracts of *Curcuma aeruginosa*, which possesses 5α-reductase inhibitory effects [41], and shampoo containing crude extracts of six different plants, which possess antioxidant, anti-inflammatory, and 5α-reductase inhibitory effects [42]. Therefore, it is likely that the active constituents included in JU7505 may lead to the improvement of AGA through changes in the physiological function of scalp skin and hair follicles.

In the present study, the levels of steroids in the hair and saliva samples were determined to assess the physiological mechanism underlying the improvement of AGA after the topical use of plant extracts, and the presence of steroid metabolic enzymes and the local stress response systems in the skin and hair follicles were considered [18,19]. We observed higher baseline hair DHT concentrations in the AGA group than the healthy controls, although the baseline hair testosterone concentrations were comparable among the examined groups. These results of the present study were consistent with other studies that reported elevated hair DHT concentrations in balding men compared with nonbalding controls [12] and comparable testosterone concentrations between balding and hairy scalp skin [13]. We also observed comparable levels of baseline Tauc<sub>hair</sub> and DHTauc<sub>hair</sub> between the examined groups. Previous studies have reported no association between circulating testosterone and DHT levels in men with AGA [43,44]. The feedforward regulation of gene expression of 5α-reductase by testosterone and DHT [45] and elevated 5α-reductase activity in the scalp skin and isolated hair roots of men with AGA compared to nonbalding men [11,46] may explain the higher DHT concentrations in the hair of men with AGA.

The baseline hair cortisol concentrations were higher in men with AGA in the treatment and placebo groups than in the healthy controls. Although information is limited regarding hair cortisol in men with AGA, the results were consistent with previous studies that reported higher concentrations of hair cortisol in samples collected from stress-exposed

**Figure 2: Hair cortisol, testosterone, dihydrotestosterone (DHT), Epi-T, dehydroepiandrosterone (DHEA) concentrations in the examined groups.** Cortisol, testosterone, DHT, Epi-T, and DHEA concentrations determined in the hair samples collected from the treatment and placebo groups and hair controls at the designated time points are presented in Fig. 2a-e, respectively.

All of the data are expressed as the mean ± standard error of the mean. Note: ns (nonsignificant) indicates p > 0.05, and *, **, and *** indicate p < 0.05, p < 0.01, and p < 0.001, respectively

**Correlations between measured steroids across the examined time points**

As presented in Table 2, significant interrelationships were observed between hair steroids. In the three examined groups, a significant correlation was observed between the baseline (t0) cortisol and testosterone concentrations (all Pearson’s p < 0.05) and between the baseline testosterone and DHT concentrations (all Pearson’s p < 0.001) (Table 2). However, neither the baseline Epi-T nor DHEA concentrations were correlated with the baseline cortisol, testosterone, or DHT concentrations in the examined groups. Significant stability was observed in the hair steroid concentrations across the examined time points in both the placebo and treatment groups. A positive relationship was observed between the hair cortisol and DHT concentrations across the three time points in the placebo group (all Pearson’s p < 0.05) and the treatment group (all Pearson’s p < 0.05) (Table 2). The hair testosterone concentration between t0 and t1 also showed a positive relationship in the placebo and treatment groups (all Pearson’s p > 0.35, p < 0.05), and the hair Epi-T concentrations between t0 and t1, between t1 and t2, and between t0 and t2 also showed a positive relationship in the treatment group (all Pearson’s p < 0.05) (Table 2).

Table 3 shows the relationship between auxiliary indices for salivary steroids secretion, that is, CARauc<sub>hair</sub> and Dauc<sub>hair</sub> in the three examined groups. Cortisol, testosterone, DHTauc<sub>hair</sub> and Dauc<sub>hair</sub> at t0. There was no inter-relationship among the indices for steroid secretion during the post-awakening period (all Pearson’s p > 0.05) (Table 3).

**DISCUSSION**

The present study examined hair-related variables and steroid levels in hair and saliva samples of AGA in men after using a formulated shampoo containing plant extracts (JU7505) to examine the physiological effect of these plant extracts. The findings of the present study were twofold: (1) the hair shaft thickness and hair density increased along receding hairlines, and (2) the concentrations of hair steroids decreased while the levels and patterns of salivary steroids did not show significant changes during the post-awakening period in men with AGA after four months of formulated shampoo use.

We observed that the treatment group had an increased hair shaft thickness and hair density along receding hairlines after four months of formulated shampoo use, indicating the improvement of AGA, with reduced hair loss and increased hair growth, by the active constituents of JU7505. The results were similar to those of previous human studies that reported the improvement of alopecia in men with AGA after using a hair tonic containing crude extracts of *Curcuma aeruginosa*, which possesses 5α-reductase inhibitory effects [41], and shampoo containing crude extracts of six different plants, which possess antioxidant, anti-inflammatory, and 5α-reductase inhibitory effects [42]. Therefore, it is likely that the active constituents included in JU7505 may lead to the improvement of AGA through changes in the physiological function of scalp skin and hair follicles.

In the present study, the levels of steroids in the hair and saliva samples were determined to assess the physiological mechanism underlying the improvement of AGA after the topical use of plant extracts, and the presence of steroid metabolic enzymes and the local stress response systems in the skin and hair follicles were considered [18,19]. We observed higher baseline hair DHT concentrations in the AGA group than the healthy controls, although the baseline hair testosterone concentrations were comparable among the examined groups. These results of the present study were consistent with other studies that reported elevated hair DHT concentrations in balding men compared with nonbalding controls [12] and comparable testosterone concentrations between balding and hairy scalp skin [13]. We also observed comparable levels of baseline Tauc<sub>hair</sub> and DHTauc<sub>hair</sub> between the examined groups. Previous studies have reported no association between circulating testosterone and DHT levels in men with AGA [43,44]. The feedforward regulation of gene expression of 5α-reductase by testosterone and DHT [45] and elevated 5α-reductase activity in the scalp skin and isolated hair roots of men with AGA compared to nonbalding men [11,46] may explain the higher DHT concentrations in the hair of men with AGA.

The baseline hair cortisol concentrations were higher in men with AGA in the treatment and placebo groups than in the healthy controls. Although information is limited regarding hair cortisol in men with AGA, the results were consistent with previous studies that reported higher concentrations of hair cortisol in samples collected from stress-exposed

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areas of skin than from unaffected areas in the same subject [34,47]. Hair cortisol concentrations are considered a manifestation of the function of the central HPA axis in the immediate past, and elevated central HPA axis functions caused by chronic stress might be associated with elevated hair cortisol concentrations [48]. It is well documented that activation of the central HPA axis by chronic stress also manifests as altered CAR values (i.e., heightened or reduced) [31]. In the present study, we observed no difference in the CAR between men with AGA and the hairy controls, which suggested no difference in the central HPA axis function between men with AGA and the hairy controls. Therefore, the results of the present and previous studies suggest that cortisol produced in the skin and hair follicles under local stress conditions likely contributes to the majority of cortisol concentrations measured in the hair.

Similar to the central HPA axis, CRH triggers a cascade of hormonal pathways that leads to the production of cortisol in the local stress response system in skin and hair follicles [18,19]. However, cortisol alone might not be the cause of hair loss in humans. For example, a hair follicle organ culture study found that treatment with CRH inhibits hair shaft elongation and hair follicle keratinocyte proliferation and stimulates premature catagen development and hair matrix...
Table 2: Correlation matrix between hair steroids

| Relationship between steroids examined at t0 (Pearson r) | Relationships of hair steroid concentrations between two examined time points (Pearson r) |
|--------------------------------------------------------|------------------------------------------------------------------------------------------|
| **1. Hairy controls**                                   |                                                                                          |
| Cortisol                                               | T DHT Epi-T DHEA                                                                           |
| Cortisol                                               | 1 0.28* 0.21 0.06 0.17                                                                  |
| T                                                      | 1 0.30* 0.20 0.15                                                                       |
| DHT                                                    | 1 0.07 0.08                                                                             |
| Epi-T                                                  | 1 0.19                                                                                  |
| DHEA                                                   | 1                                                                                       |
| **2. Placebo group**                                   |                                                                                          |
| Cortisol                                               | T DHT Epi-T DHEA                                                                           |
| Cortisol                                               | 1 0.48* 0.24 0.14 0.22                                                                  |
| T                                                      | 1 0.41* 0.17 0.19                                                                       |
| DHT                                                    | 1 0.21 0.15                                                                             |
| Epi-T                                                  | 1 0.07                                                                                  |
| DHEA                                                   | 1 DHEA 0.60 0.13 0.17                                                                   |
| **3. Treatment group**                                 |                                                                                          |
| Cortisol                                               | T DHT Epi-T DHEA                                                                           |
| Cortisol                                               | 1 0.33* 0.26 0.16 0.14                                                                  |
| T                                                      | 1 0.43* 0.24 0.02                                                                       |
| DHT                                                    | 1 0.27 0.07                                                                             |
| Epi-T                                                  | 1 0.15                                                                                  |
| DHEA                                                   | 1 DHEA 0.01 0.22 0.03                                                                   |

**Table 3: Correlation table between salivary steroid concentrations**

| Relationship between steroids examined at t0 (Pearson r) | **Hairy controls**                                                                 |
|--------------------------------------------------------|-----------------------------------------------------------------------------------|
| CArauc                                                 | Tauc<sub>awk</sub> DHTauc<sub>awk</sub> EipTauc<sub>awk</sub> Dauc<sub>awk</sub> |
| CArauc                                                 | 1 0.21 0.15 0.10 0.18                                                              |
| Tauc<sub>awk</sub>                                      | 1 0.29 0.18 0.14                                                                  |
| DHTauc<sub>awk</sub>                                   | 1 0.16 0.05                                                                       |
| EipTauc<sub>awk</sub>                                  | 1 0.22                                                                          |
| Dauc<sub>awk</sub>                                     | 1                                                                 |

| **Placebo group**                                       |                                                                                   |
| CArauc                                                 | Tauc<sub>awk</sub> DHTauc<sub>awk</sub> EipTauc<sub>awk</sub> Dauc<sub>awk</sub> |
| CArauc                                                 | 1 -0.16 0.11 -0.04 0.18                                                          |
| Tauc<sub>awk</sub>                                      | 1 0.30 -0.12 0.11                                                               |
| DHTauc<sub>awk</sub>                                   | 1 0.11 0.31                                                                     |
| EipTauc<sub>awk</sub>                                  | 1 0.06                                                                         |
| Dauc<sub>awk</sub>                                     | 1                                                                 |

| **Treatment group**                                     |                                                                                   |
| CArauc                                                 | Tauc<sub>awk</sub> DHTauc<sub>awk</sub> EipTauc<sub>awk</sub> Dauc<sub>awk</sub> |
| CArauc                                                 | 1 0.02 0.07 0.11 0.16                                                           |
| Tauc<sub>awk</sub>                                      | 1 0.27 0.16 -0.15                                                               |
| DHTauc<sub>awk</sub>                                   | 1 0.22 -0.07                                                                    |
| EipTauc<sub>awk</sub>                                  | 1 -0.10                                                                        |
| Dauc<sub>awk</sub>                                     | 1                                                                 |

DHEA: Dihydrotestosterone

In addition to the improvement of AGA, we also observed a concomitant reduction in the hair cortisol and DHT concentrations but did not observe changes in the levels of salivary steroids in men with AGA after 4 months using the formulated shampoo. These results implied that topical use of the plant extracts reduced the stress burden imposed on scalp skin and hair follicles. Until now, information on the factors that activate the local stress response system has been limited. A possible explanation for our observation is that the expression levels of CRH, CRH-R, and POMC mRNAs in epidermal keratinocytes, melanocytes, and sebaceous glands are stronger in inflammatory lesional skin than in normal skin [51], indicating that inflammatory cytokines upregulate the de novo synthesis of cortisol in the local stress response system. Indeed, cortisol biosynthesis in epidermal keratinocytes is reported to be enhanced by interleukin-1β (IL-1β) [20]. A recent study showed that exogenously added CRH enhances the synthesis of CRH-receptor 1 and 2, ACTH, melanocortin receptor 2, and TGF-β2 but reduces hair shaft elongation in biopsied hair follicles from balding vertex scalp areas of men with AGA [52]. The expression of TGF-β2, a local mediator of cellular responses to steroids, is known to be upregulated in the hair follicles by testosterone and DHT [53,54]. Therefore, it is speculated that the active constituents included in JU7505 may play a role in reducing the stress burden, such as inflammation in scalp skin and hair follicles, which results in a concomitant decrease in the concentrations of hair steroids, including cortisol and DHT, in men with AGA.

In previous studies, we observed a marked decrease in plasma DHEA concentrations and a concomitant increase in cortisol concentrations in men with AGA [18]. The cortisol increase was not due to changes in the cortisol/cortisone ratio, which suggests that the increase in cortisol is a primary response to inflammation. In addition, the decrease in DHEA levels is consistent with the findings of previous studies indicating a decrease in the relative abundance of progestins and androgens in the peripheral organs [50].

In conclusion, the results of this study support the hypothesis that the active constituents included in JU7505 may play a role in reducing the stress burden, such as inflammation in scalp skin and hair follicles, which results in a concomitant decrease in the concentrations of hair steroids, including cortisol and DHT, in men with AGA. Further studies are required to elucidate the molecular mechanisms underlying the anti-inflammatory and anti-keratinocyte apoptosis effects of JU7505.
in the present study. However, previous findings indicated that the burden of stress, such as microbial-induced inflammation in scalp skin and hair follicles, might be reduced by the actions of the constituents included in JU7505, which manifested as a decrease in the hair cortisol concentrations in the treatment group.

The present study has some limitations that should be acknowledged. First, the number of participants was relatively small to more easily evaluate the beneficial effects of the plant extracts. A statistically significant difference was observed between the treatment and placebo groups in each category of examination. However, the number of participants in each subgroup analysis was relatively small, which may have limited the power of the significant difference in this study. Therefore, the findings of this study need to be confirmed in more extensive studies with a higher sample size. Second, we observed the beneficial effects of plant extracts on AGA in the present study, which represents an essential preliminary step toward preparing more effective plant extracts for AGA treatment.

CONCLUSION

Hair-related variables are commonly used as a primary outcome measure in studies on AGA in men. To our knowledge, this is the first trial examining the physiological mechanisms underlying the improvement of AGA after the topical use of plant extracts. The present study observed that AGA in men was associated with elevated hair cortisol and DHT concentrations rather than post-awakening salivary cortisol and DHT levels, suggesting that AGA may be associated with the activation of the local stress response system of scalp skin and hair follicles, which provides precursor steroids for DHT formation. In addition, the results showed that men with AGA presented increased hair shaft thickness and hair density after the treatment; moreover, hair loss was reduced, hair growth was increased, and hair steroid concentrations, including cortisol and DHT concentrations, were decreased after using the formulated shampoo containing JU7505, a mixture of extracts of six different plants that were reported to have anti-inflammatory, antimicrobial, antioxidant, cortisol, or 5α-reductase inhibitory properties in other previous studies. However, changes in the levels and patterns of salivary steroids were not observed after the treatment. The present study was not focused on identifying the active constituents present in the plant extract JU7505. However, the results indicated that the active constituents in JU7505 might have beneficial effects on AGA, which led to improvements in AGA symptoms, hair loss prevention, and hair growth effects. In addition, the reduced concentrations of hair steroids, including cortisol, DHT, testosterone, Epi-T, and DHEA, indicated that the extracts had a reducing effect on steroidogenic activities in scalp skin and hair follicles. Therefore, topical applications of such extracts, such as shampoo and hair tonic, containing anti-inflammatory, antioxidant, antimicrobial, cortisol, and 5α-reductase inhibitory properties would be helpful for men with AGA.

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AUTHORS’ CONTRIBUTIONS

All authors contributed equally.

CONFLICTS OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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