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ORIGINAL ARTICLE

A proprietary lipidosterolic extract of Serenoa repens promotes hair growth through mechanisms that extend beyond 5-alpha reductase inhibition: Insights from human hair follicle organ culture

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Abstract

While androgenetic alopecia (AGA) is primarily driven by excessive 5-α reductase (5-αR) activity, further mechanisms also contribute to the development of AGA and other hair loss disorders. Here, we explored the properties of the proprietary lipidosterolic extracts of the plant *Serenoa repens* (LSESr), also known as Saw Palmetto, focusing on USPlus® DERM Bioactive Fatty Acids (USPlus® DERM). USPlus® DERM contains concentrated levels of bioactive free fatty acids (FFAs) that are integral lipids found in the hair shaft and modulate pathways relevant to hair follicle (HF) function. Therefore, USPlus® DERM promises to have both 5-αR-dependent and also -independent hair growth-promoting effects. Here, we initially confirmed the 5-αR inhibitor activity of USPlus® DERM in primary human hair follicle dermal papilla cells. USPlus® DERM exhibited a more potent 5-αR inhibition than a standard, commercially available saw palmetto extract and a standardized LSESr meeting the US Pharmacopoeia monograph, with IC_{50} values of 0.39, 29.1 and 9.1 μg/mL respectively. To explore potential 5-αR-independent responses, USPlus® DERM was administered at two different concentrations, 0.4 and 10 μg/mL, to androgen-independent, 'clinically healthy' full-length HFs ex vivo obtained from the occipital scalp of male donors, in the absence of testosterone. Interestingly, USPlus® DERM at 0.4 μg/mL significantly reduced the number of melanin clumps, regarded as signs of organ culture mediated stress conditions. Despite inter-donor variations, USPlus® DERM prolonged anagen ex vivo, particularly at the low concentration, evidenced by a significant reduction in the hair cycle score and the tendency to boost hair matrix keratinocyte proliferation (Ki-67$^+$cells). USPlus® DERM did not affect dermal papilla inductivity, as measured by versican expression and alkaline phosphatase activity. While the percentage of K15$^+$ epithelial HF stem cells (eHFSC) remained

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unaffected, 0.4 µg/mL USPlus® DERM tendentially enhanced K15 expression and reduced the number of Ki-67$^+$K15$^+$ cells, indicating reinforcement of the eHFSCs niche. These preliminary findings suggest that USPlus® DERM has the potential to promote hair growth and to enhance HFSC stemness in the 'clinically predictive' HF organ culture model, independently from 5-αR inhibition. Thus, USPlus® DERM deserves to be further investigated as an anti-hair loss strategy, for not only AGA management but also other hair loss disorders.

KEYWORDS

claim substantiation, free fatty acids, hair growth, hair treatment, saw palmetto extract, *Serenoa repens*

Résumé

Si l'alopécie androgénétique (AAG) est principalement due à une activité excessive de la 5-alpha-réductase (5-alpha-R), d'autres mécanismes contribuent également au développement de l'AAG et à d'autres troubles de la perte de cheveux. Nous avons exploré ici les propriétés des extraits lipidostéroliques exclusifs de la plante *Serenoa repens* (LSESr), également connue sous le nom de Saw Palmetto, en nous concentrant sur les acides gras bioactifs USPlus® DERM (USPlus® DERM). L'USPlus® DERM contient des niveaux concentrés d'acides gras libres (AGL) bioactifs qui sont des lipides intégraux présents dans la tige des cheveux et modulent les voies pertinentes pour la fonction des follicules pileux (FP). Par conséquent, l'USPlus® DERM promet d'avoir des effets favorisant la croissance des cheveux à la fois dépendants et indépendants du 5-alphaR. Ici, nous avons initialement confirmé l'activité inhibitrice du récepteur 5 alpha-R de l'USPlus® DERM dans les papilles papillaires folliculaires primitives humaines. L'USPlus® DERM a montré une inhibition plus puissante de la 5-alphaR qu'un extrait de Saw Palmetto standard disponible dans le commerce et un LSESr standardisé conforme à la monographie de la Pharmacopée américaine, avec des valeurs CI_{50} de 0,39 microgrammes/ml, 29,1 microgrammes/ml et 9,1 microgrammes/ml respectivement. Afin d'explorer les réponses potentielles indépendantes du 5-alphaR, l'USPlus® DERM a été administré à deux concentrations différentes, 0,4 et 10 microgrammes/ml, à des IC de longueur complète, non dépendantes des androgènes et cliniquement saines, *ex vivo* obtenues à partir du cuir chevelu occipital de donneurs masculins, en l'absence de testostérone. Il est intéressant de noter que l'USPlus® DERM à 0,4 microgramme/ml a significativement réduit le nombre d'amas de mélanine, considérés comme des signes de conditions de stress induites par la culture d'organes. Malgré la variation inter-donneurs, l'USPlus® DERM prolonge l'anagène *ex vivo*, en particulier à faible concentration, mise en évidence par une réduction significative du score du cycle capillaire et la tendance à stimuler la prolifération des kératinocytes de la matrice pileuse (cellules Ki-67$^+$). USPlus® DERM n'a pas affecté l'inductivité papillaire cutanée, telle que mesurée par l'expression du versican et l'activité de la phosphatase alcaline. Alors que le pourcentage de cellules souches épithéliales de l'IC K15$^+$ (eHFSC) n'était pas affecté, l'USPlus® DERM 0,4 microgrammes/ml améliorait généralement l'expression de K15 et réduisait le nombre de cellules Ki-67$^+$K15$^+$,

ce qui indique un renforcement de la niche des eHFSC. Ces résultats préliminaires suggèrent que l'USPlus® DERM a le potentiel de favoriser la pousse des cheveux et d'améliorer la souche HFSC dans le modèle de culture d'organe HF « cliniquement prédictif », indépendamment de l'inhibition du 5-alphaR. Ainsi, USPlus® DERM mérite d'être étudié de manière plus approfondie en tant que stratégie anti-perte de cheveux, non seulement pour la prise en charge de l'AAG, mais aussi pour d'autres troubles liés à la perte de cheveux.

INTRODUCTION

Androgenetic alopecia (AGA), the overarching term for androgen-related hair loss in both men and women, is a common and progressive form of hair loss that affects millions of individuals globally [1–4]. While patterned hair loss in men is primarily associated with androgens, female pattern hair loss is only related to androgen levels when associated with hyperandrogenism but can have a great variety of other causes [5, 6]. AGA is characterized by the miniaturization and thinning of scalp hair follicles (HFs), ultimately leading to a reduction in hair density and baldness [4]. The underlying mechanisms of AGA are complex and multifactorial, involving a combination of genetic predisposition [4, 7, 8], environmental factors such as diet or stress [4, 9] and hormonal influences [4, 10, 11].

Particularly, the increased conversion of testosterone, and to a lesser extent dehydroepiandrosterone (DHEA), to dihydrotestosterone (DHT) by 5-α reductase (5αR) is a hallmark of AGA [4–6]. DHT binds to the androgen receptor (AR), a ligand-dependent nuclear transcription factor [12] primarily found within the dermal papilla (DP) [13]. The DHT–receptor complex binds to β-catenin, preventing it from promoting the transcription of Wnt molecules, which are essential for stimulating the proliferation of hair matrix keratinocytes. This, in turn, shortens the anagen phase and prolongs the telogen phase [14, 15], leading to increased and extended hair shedding. Interestingly, AR expression is reported to be upregulated in frontal/vertex compared to occipital HFs, where AGA develops [16]. Indeed, occipital HFs are recognized to be androgen-independent [4, 17] or (most likely erroneously) androgen-insensitive [4, 18]. This terminology highlights a crucial distinction: unlike HFs located in the frontal and vertex regions, occipital HFs remain unaffected by androgen-induced hair cycle changes. In other words, androgens do not facilitate the anagen-to-catagen transition in these HFs, and thus do not contribute to hair loss [17].

As of now, the selective 5αR inhibitor finasteride is one of the only two drugs approved by the FDA for the treatment of AGA [10, 11]. Yet, controversial data report adverse effects under finasteride treatment, such as a reduced libido and even sometimes lasting erectile dysfunction called 'post finasteride syndrome' [19, 20]. Additionally, patients express concerns about the long-term use of the drug, which can be associated with psychiatric symptoms [21, 22]. Therefore, the development of natural treatment options with high efficacy and lower side effects is highly sought after.

In this regard, saw palmetto (*Serenoa repens*), which has gained recognition as an effective nutraceutical for the symptomatic treatment of prostatic conditions, including benign prostatic hypertrophy [23], is increasingly being explored for its role in managing androgen-dependent hair loss. Indeed, clinical studies demonstrated that oral and/or topical application of saw palmetto extracts can address hair loss, in association with minor side effects, and patients have reported high rates of satisfaction [24–26]. Saw palmetto extracts can vary in lipid composition and activity depending on the extraction process. Due to the unique fatty acid profile, they are potent inhibitors of the two 5αR isoforms, 5αR-1 and -2. Among these extracts, Permixon® is recognized as a leading European herbal medicine, known for its high concentration of total and free fatty acids (FFAs) [27]. Notably, USPlus® DERM, which is extracted from saw palmetto berries using the DeepExtract® proprietary, ultrahigh-pressure carbon dioxide extraction technology, with additional FFA conversion and fractionation, demonstrates even higher FFA levels [28]. A recent study demonstrated that topical application of USPlus® DERM serum (2%) improved hair growth and hair density in males and females suffering from unresolved hair loss [29]. These positive outcomes are most likely in part caused by saw palmetto's inhibitory effects on 5αR [28], thereby slowing the progression of hair loss. However, further evidence is required to fully support this evidence [25, 30].

Additionally, preclinical studies are needed to clarify whether saw palmetto extracts—including USPluS® DERM—can also have also 5αR-independent effects on hair growth. The presence of FFAs in saw palmetto could support the FFA balance prevalent in hair, thereby contributing to the hydrophobic characteristics that maintain hair health and strength [31]. FFAs, particularly linoleic, oleic and palmitic acids, are a major component of the cuticle, medulla and cortex of the hair shaft [32].

Additionally, FFAs have been indicated to promote Wnt signalling [33], have anti-microbial, anti-oxidant and anti-inflammatory properties [34], and stimulate collagen production, which all could aid in skin and hair health [35]. Of note, fatty acid metabolism is downregulated in the affected scalp of male AGA patients [36], and a lower lipid content and composition have been found in the sebum of AGA patients [37]. Additionally, a reduced abundance of FFAs was observed in occipital HFs from FPHL patients [38]. All of this further suggests that FFAs have effects on hair growth that extend beyond 5αR inhibition.

Objective

In this preliminary study, we aimed to compare the 5αR inhibitory potential of the new, proprietary USPlus® DERM with other saw palmetto extracts and finasteride in primary human dermal papilla cells (HFDPCs) in vitro. Additionally, we primarily sought to explore the 5αR-independent properties of USPlus® DERM using the gold standard preclinical, clinically predictive [39, 40] HF organ culture model [41]. To exclude any 5αR-dependent effect, we implemented two key strategies: (1) We omitted testosterone addition during the culture and (2) utilized 'androgen-independent' healthy full-length HFs obtained from the occipital scalp of male donors.

MATERIALS AND METHODS

Donor material and ethics consideration

Human full-length HFs were micro-dissected from follicular unit extraction (FUE) obtained from six male donors (25, 41, 41, 51, 56, 63 years) as previously described [42]. All human scalp skin specimens were obtained after informed, written consent under the QIMA Monasterium Biobank approval (2019-297-f-S) and processed for further analysis under ethics committee study approval (2020-954-f-S), University of Muenster (2015-602-f-S), the Marmara University School of Medicine clinical research ethics committee (09.2023.830), and the Comité de Bioética de la Universidad Fernando Pessoa Canarias (032020 06 22). All experiments on human tissue were performed according to the study plan 2020-954-f-S and the Declaration of Helsinki principles.

HFDPC culture

Human follicle dermal papilla cells (HFDPCs) were purchased from Promocell (Heidelberg, Germany). The cells were maintained in DMEM (Fisher Scientific, Illkirch, France) + 10% FCS (Biowest, Nuaillé, France) until use. All experiments were performed at Passage 7. HFDPCs were seeded in 24-well plates and were allowed to adhere to the bottom of the wells for 24 h in DMEM + 10% FCS. Afterwards, the culture medium was replaced by DMEM + 1% FCS containing either finasteride (10 μM, positive control), with a commercially available saw palmetto extract (SPE), a standardized LSESr meeting the US Pharmacopoeia monograph (LSESr), or USPlus® DERM (at concentrations of 0.1, 0.3, 1, 3, 10, 30, 60 and 100 μg/mL) for 24 h. These three extracts differ in their proportion of FFAs to total fatty acids, which are 71%, 76% and 91% for SPE, LSESr and USPlus DERM, respectively. Thereafter, the treatments were renewed and 0.051 μCi per well of [^{14}C]-testosterone (Revvity, Bussy Saint Martin, France) was added and the cells were incubated for another 24 h. Afterwards, the culture supernatants were collected for testosterone metabolism analysis and the cells were used to assess viability using a standard WST-8 assay. All experimental conditions were performed in technical replicates of $n = 3$.

Cytotoxicity in HFDPCs

The cytotoxicity of SPE, LSESr and USPlus® DERM was determined in HFDPCs using a WST-8 assay following the manufacturer's instructions (Abbkine Scientific, Atlanta, Georgia, USA). Briefly, cells were incubated with 30 μL of the WST-8 substrate (prediluted at 1/10) for 2 h at 37°C with 5% CO_2. Afterwards, the supernatant was collected, and the absorbance at 450 nm was evaluated using a spectrometer (VERSAmax, Molecular Devices).

Steroid extraction and analysis

Testosterone metabolism was assessed as described before [43]. The steroid molecules were extracted from supernatants with a chloroform/methanol mix (98:2) (bought from Sigma-Aldrich, Saint Quentin Fallavier, France and Fisher, respectively). Briefly, in Pico Provial 4-mL tubes (Perkin), 400 μL of the extraction mix were mixed with 200 μL supernatants, and the tubes were vortexed for 15 s. Then, the tubes were centrifuged (4 min, 700 g at room temperature), and 300 μL of the inferior organic phase was collected and was evaporated under an air stream for at least 1 h. After evaporation, the pellets were solubilized with 30 μL dichloromethane (Fisher), and the different molecular species (testosterone metabolites: mainly testosterone, DHT androstenedione, 4-androstene-3, 17-dione and α-androstane-3α, 17β-diol), were separated by thin layer chromatography

(TLC) and using a solvent system containing dichloromethane, ethyl acetate, and methanol (82.5:14.6:2.9) (all from Fisher). Autoradiography was performed on TLC plates using a Typhoon (Amersham™ Typhoon™ IP Biomolecular Imager—GE Healthcare Life Sciences), and the level of testosterone and DHT was estimated by densitometric analysis of the different spots corresponding to testosterone metabolites (ImageQuantTL software—Cytiva, Saint Germain En Laye, France).

Hair follicle organ culture and treatment

Human full-length anagen VI hair follicles (HFs) were isolated from the occipital scalp region of six clinically healthy male Caucasian donors aged 25–63 years (mean age 46.17 y/o). The follicles were cultured at 37°C with 5% CO_2 in William's Complete Medium (WCM), as previously described [42, 44]. After a resting period of 24 h, each HF was independently assessed by at least two examiners to confirm the anagen stage and ensure D0–D1 growth of ≥2%. Only qualified follicles were included in the study and were randomly allocated to experimental groups, ensuring comparable average D0–D1 growth percentages across groups.

Throughout the experiment, each group was assigned an identification number (e.g. #1, #2) and handled blinded by a designated technician. HFs were treated ex vivo for 5–9 days with either 0.4 or 10 µg/mL USPlus® DERM, or vehicle control (0.08% DMSO) in WCM, with treatments replaced bi-daily.

A culture period of 5–9 days was used in this study, as it is well-established, widely accepted, and sufficient for assessing anagen maintenance and prolongation as well as hair shaft production in human HF organ culture [42, 44]. Moreover, this period has previously demonstrated reproducible and clinically relevant results [39, 40, 45].

At the end of culture, HFs were embedded in Cryomatrix (OCT) and snap-frozen in liquid nitrogen for further analysis. All result analyses were performed by a separate individual who was blinded to the group assignments.

Immunofluorescence stainings and (immuno-)histomorphometry

Cryosections of 6 µm were prepared with a Leica cryostat (Leica Biosystems, Wetzlar, Germany); consecutive sections of each HF were collected, and slides were stored at −80°C [42].

To stain apoptotic and proliferating cells in the hair matrix, a Ki-67/TUNEL double staining was performed using the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore) in combination with a mouse anti-Ki-67 staining as described previously [39, 44]. Briefly, after fixation with 4% PFA and pre-treatment with the ApopTag solutions for the TdT-Enzyme, cryosections were blocked with goat normal serum and incubated overnight at 4°C with a mouse anti-Ki-67 antibody (1:800 in PBS; Cell Signalling Technology). Ki-67 antibody was detected with a goat anti-mouse IgG rhodamine secondary antibody (1:200; Jackson ImmunoResearch), and apoptotic cells were detected with a Fluorescent-labelled Anti-Digoxigenin antibody.

To analyse versican expression, cryosections were incubated with mouse anti-human versican (1:400, DSHB) overnight at 4°C, which was detected with a secondary goat anti-mouse Alexa Fluor 488 antibody (1:400, Invitrogen). Versican intensity was analysed in the DP [39, 46].

Epithelial HF stem cell (eHFSC) expression and proliferation were examined as described before [46–48]. In short, cryosections were incubated overnight at 4°C with mouse anti-K15 (1:500, LHK15; Abcam, Cambridge, UK) and rabbit anti-Ki-67 (1:100, Abcam, Cambridge, UK) and visualized using appropriate Alexa Fluor secondary antibodies (Thermo Fisher, UK). Fluorescence intensities for K15 and positive cell numbers for K15 and Ki-67 were quantified in the HF bulge.

Counterstaining with DAPI was performed during every staining protocol to visualize nuclei.

Images were taken using a Keyence fluorescence microscope (BZ9100 Keyence, Osaka, Japan) maintaining a constant set exposure time throughout imaging for further analysis. Staining immunoreactivity or the number of positive cells was analysed in standardized reference areas using the software ImageJ (National Institutes of Health, Bethesda, MD, USA, open source).

Masson Fontana histochemistry and melanin clumping analysis

For the histochemical visualization of melanin, the Masson–Fontana staining was performed as previously described [49, 50]. Melanin pigments were stained as black dots using Masson Fontana; the presence of abnormal large melanin clumping (i.e. melanin positive conglomerates that were larger than keratinocyte nuclei) was counted in the HM up to 10 lines of cells above the end of the dermal papilla [51, 52].

Alkaline phosphatase activity in situ

Alkaline phosphatase activity in situ was detected using the Vector® Blue Substrate Kit and alkaline phosphatase (SK5300, Biozol) according to the manufacturer's instructions, with levamisole as a negative control [39, 53]. Alkaline phosphatase intensity was measured in the DP.

Hair cycle staging and scoring

Microscopic hair cycle staging was performed at the end of the culture on the basis of morphology, Masson–Fontana histochemistry, and Ki-67/TUNEL immunostaining, as previously described [44]. Hair cycle scoring was calculated using a standardized, arbitrary score (anagen = 100; catagen = 200; early catagen = 300; mid-catagen = 400) [54]. Thus, a lower score means that the HFs are more established in anagen, and a higher score means that the HFs are more progressed to catagen.

Statistical analysis

Statistical analyses were performed using Graphpad Prism 9 (GraphPad Software Inc.). Data were tested for normal distribution using the D'Agostino and Pearson omnibus normality test. Two groups were compared using the Mann–Whitney U test $*p < 0.05$ and three groups using the Kruskal–Wallis test with Dunn's multiple comparisons $^{\#}p < 0.05$. Data are expressed as mean ± SEM.

RESULTS

USPlus® DERM demonstrates greater 5α-reductase inhibition in HFDPCs than other saw palmetto extracts, but its potency does not reach that of finasteride

We first compared the 5αR-inhibitory potential of a commercially available saw palmetto extract (SPE), a standardized LSESr meeting the US Pharmacopoeia monograph (LSESr), USPlus® DERM, or finasteride, in human HFDPCs in vitro. The three extracts differ in their proportion of FFAs to total fatty acids, which are 71%, 76% and 91% for SPE, LSESr and USPlus DERM respectively. All three extracts maintained relatively high viability of HFDPCs across a concentration range of 0.14 to 100 μg/mL (Figure 1a). A marginal reduction in HFDPC viability of approximately 10%, 15%, or 25% was observed at a concentration of 300 μg/mL for SPE, LSESr and USPlus® DERM, respectively (Figure 1a). Application of 100 μg/mL of SPE, LSESr or USPlus® DERM resulted in 67%, 79% and 85% inhibition of 5αR, respectively, compared to 95% inhibition by finasteride (Figure 1b). Furthermore, USPlus® DERM exhibited the greatest potency in inhibiting 5αR activity at lower concentrations compared to the other two extracts (Figure 1b–d). Specifically, the IC50 value for USPlus® DERM was determined to be less than 0.39 μg/mL, while SPE and LSESr displayed IC50 values of 29.1 and 9.1 μg/mL, respectively, indicating an almost 75-fold increase in inhibitory efficacy of USPlus® DERM relative to the commercially available saw palmetto extract (Figure 1c). However, it is important to note that the inhibitory capacity of USPlus® DERM reached a plateau as concentrations increased to 100 μg/mL, demonstrating converging levels of inhibition beyond this threshold (Figure 1b–d). These results indicate that USPlus® DERM outperforms the other two extracts and has the highest potency to inhibit 5αR in vitro.

USPlus® DERM prolongs anagen in human 'clinically' healthy occipital HFs ex vivo

USPlus® DERM contains high levels of FFAs, which have been implicated in various biological pathways that influence HF function [31–34], indicating a potential beneficial role of USPlus® DERM on hair growth that goes beyond 5αR inhibition. Therefore, we treated androgen-independent 'clinically' healthy full-length HFs obtained from the occipital scalp of male donors with USPlus® DERM [42] in the absence of testosterone to assess potential 5αR-independent effects. We found no concentration-induced cytotoxicity of USPlus® DERM in human HFs (Figure 2a,b), as assessed by the number of melanin clumps [52, 55]. Notably, treatment with 0.4 μg/mL USPlus® DERM even significantly reduced melanin clump formation (Figure 2b). Moreover, microscopic analysis revealed that both concentrations of USPlus® DERM prolonged the anagen phase, although inter-donor variations were observed in the magnitude of such response (Figure 2c,d). Interestingly, the anagen-prolonging effect was more consistent and thus more prominent with the lower dose of USPlus® DERM, confirmed by a significant decrease in the hair cycle score (Figure 2e), which is calculated by attributing an arbitrary score to each hair cycle phase where a lower score indicates that more HFs are in the anagen phase [44]. Treatment with 0.4 μg/mL USPlus® DERM also sustained the proliferation of keratinocytes in the germinative hair matrix, supporting the anagen maintenance (Figure 2f). This increase did not persist when only HFs remaining in anagen at the end of the culture were included in the assessment. Additionally, the

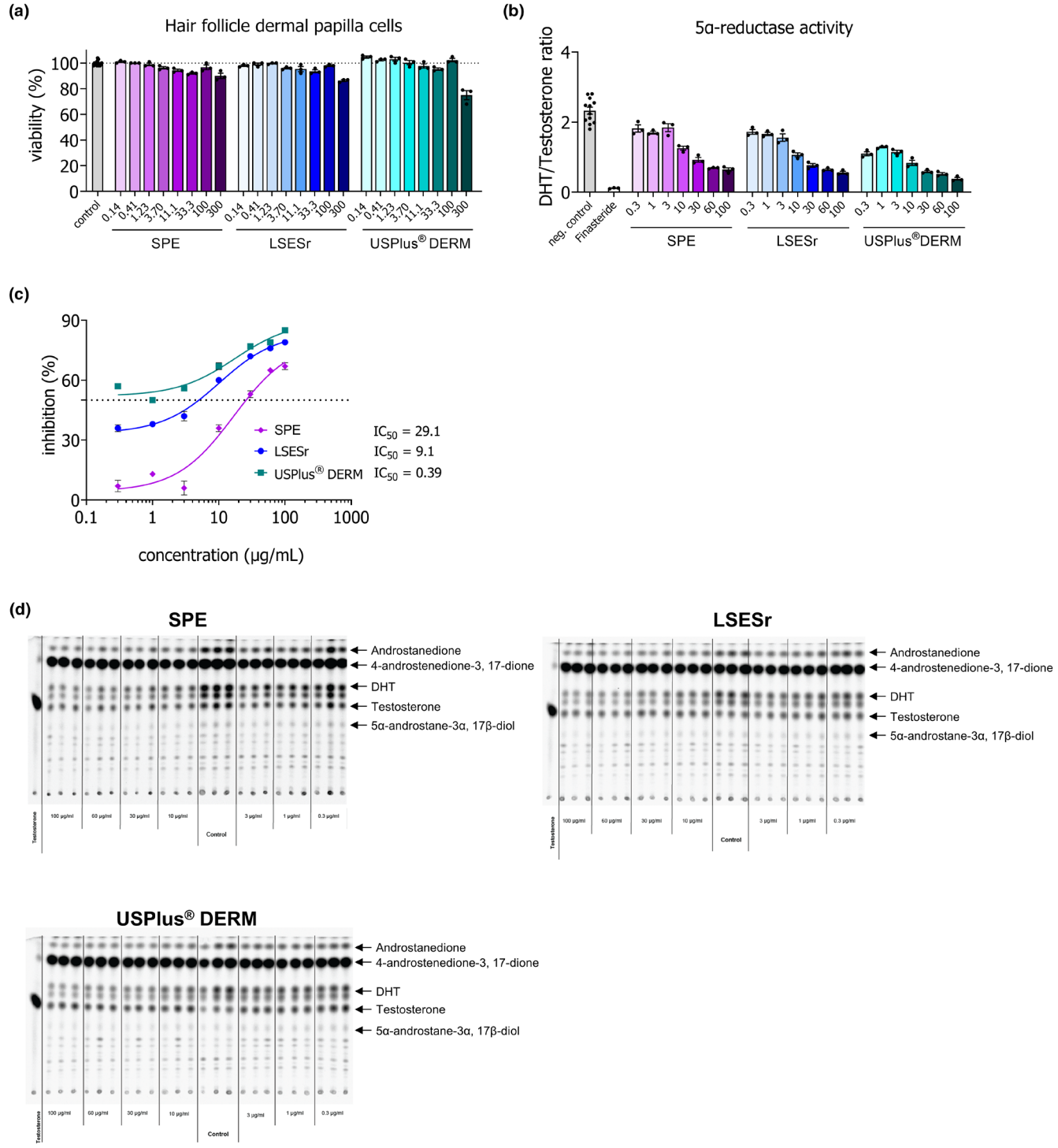

FIGURE 1 USPlus® DERM does not affect HFDPC viability and inhibits 5α-reductase activity in HFDPCs more efficiently than a commercially available saw palmetto extract (SPE) and a standardized LSESr meeting USP monograph (LSESr). (a) Percentage viability of hair follicle dermal papilla cells (HFDPC) treated with 0.14–300 µg/mL of SPE, LSESr or USPlus® DERM for 48 h. (b) Quantification of DHT/testosterone ratio in HFDPCs following treatment with vehicle control, 10 µM Finasteride, or 0.3–100 µg/mL of SPE, LSESr or USPlus® DERM for 48 h, indicating their 5α-reductase (5αR) inhibitory potential. (c) IC_{50} determination of mL of SPE, LSESr or USPlus® DERM on 5α-reductase activity in HFDPCs. (d) Representative images of thin layer chromatography and autoradiography showing the abundance of different testosterone metabolites. Data are presented as mean ± SEM. $n = 3$ technical replicates from 1 culture. DHT, Dihydrotestosterone.

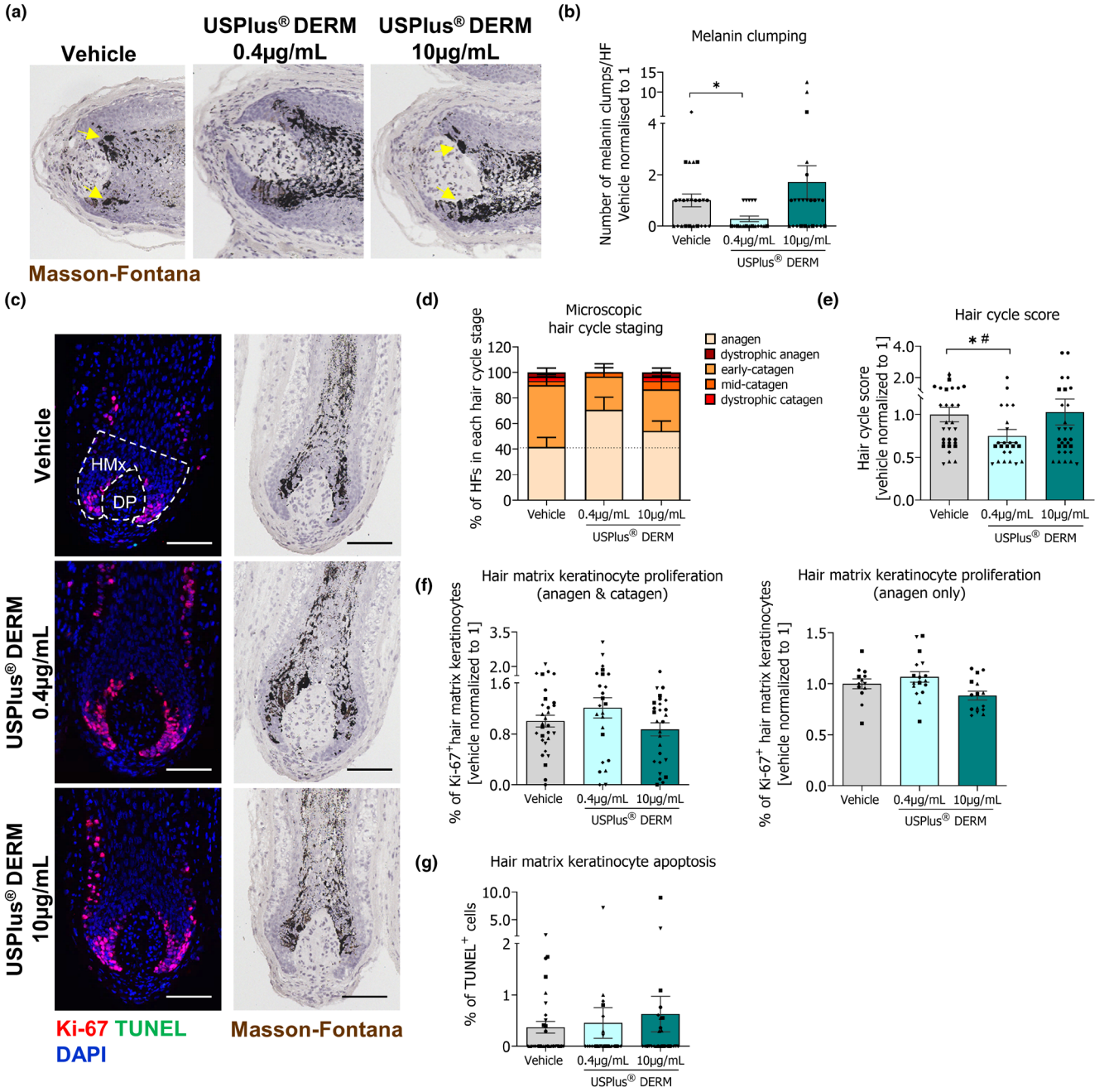

FIGURE 2 USPlus® DERM prolongs the anagen phase in healthy human HFs ex vivo. Hair follicles (HFs) were treated with vehicle control or USPlus® DERM (0.4 or 10 µg/mL) for 5–9 days ex vivo. (a) Representative images and (b) quantification of melanin clumping. $n = 18–24$ HFs/group from five to six healthy donors. (c) Representative images of Ki-67/TUNEL and Masson Fontana staining. (d) Microscopic hair cycle staging, $n = 5–6$ donors. (e) Hair cycle score. $n = 24–31$ HFs/group from five to six healthy donors. (f) Quantification of hair matrix keratinocyte proliferation (Ki-67$^+$ cells), $n = 24–30$ HFs/ group (anagen & catagen HFs) or 13–17 HFs/ group (only anagen) from five to six healthy donors. (g) Quantification of hair matrix keratinocyte apoptosis (TUNEL$^+$ cells) in anagen & catagen HFs, $n = 24–30$ HFs/ group from five to six healthy donors. All data are presented as mean ± SEM. Dots of different shape indicate different donors. D'Agostino & Pearson omnibus normality test, no Gaussian distribution; Kruskal–Wallis test, with Dunn's multiple comparison test—vehicle fixed, # $p < 0.05$ or Mann–Whitney test—vehicle fixed, *$p < 0.05$. Scale bars = 100 µm. DP: DERM al papilla, HMx: Hair matrix.

apoptosis rate (TUNEL+ cells) in HM keratinocytes was unaffected upon treatment with both concentrations of USPlus® DERM (Figure 2g). These results underscore the potential of USPlus® DERM to positively affect hair health and growth also in the absence of testosterone, indicating 5αR-independent effects.

USPlus® DERM does not affect DP inductivity, but reinforces the eHFSC stem cell niche in healthy human HFs ex vivo

We further investigated the mechanisms underlying the anagen-prolonging effect of USPlus® DERM in healthy human occipital HFs ex vivo. Given that DP inductivity orchestrates the hair cycle phases through interaction with HM keratinocytes [54, 56], is closely affiliated with Wnt signalling [56], a pathway known to be regulated by free fatty acids (FFAs) [33], and is involved in HF miniaturization, we assessed whether USPlus® DERM could potentially impact DP inductivity. This was further encouraged by a recent study showing that a lipid mixture containing FFAs enhanced inductivity in DP cells in vitro and elongated hair shaft-like structures of HF organoids [36]. Therefore, we assumed similar effects for USPlus® DERM. However, USPlus® DERM had no clear impact on either alkaline phosphatase activity or the expression of versican, both of which serve as markers for DP inductivity (Figure 3a–c).

Next, we examined the expression and proliferation of eHFSCs, which are vital for maintaining HF structure and function by serving as a reservoir for the cells required to sustain the HF epithelium [57]. Furthermore, eHFSCs are essential for the generation of new hair shaft and inner root sheath cells during anagen, thereby facilitating the production of new hair, and their dysregulation is involved in HF miniaturization [57]. Application of 0.4 µg/mL USPlus® DERM did not influence the number of $K15^+$ eHFSCs; however, it did elevate $K15^+$ expression and, meanwhile, reduced eHFSC proliferation (Figure 3d,e). Thus, the increase in $K15^+$ expression indicates a reinforcement of the stem cell niche [58], while reduced proliferation points to the maintenance of eHFSC quiescence [59], both of which are critical mechanisms of eHFSC resilience and essential for sustained hair cycle induction and anagen maintenance [60].

DISCUSSION

The use of natural products for addressing hair loss has significantly expanded in recent years, following a broader movement that values holistic health and well-being. This trend aligns with a growing awareness of the potential side effects associated with synthetic drugs [61, 62]. Our preliminary results demonstrate that the new, proprietary, natural extract of *Serenoa repens*, USPlus® DERM Bioactive Fatty Acids, efficiently inhibits 5αR activity in HFDPCs in vitro, thus preventing the conversion of testosterone to the more potent androgen DHT. Additionally, it prolongs the anagen phase ex vivo in androgen-independent 'clinically healthy' full-length HFs from the occipital scalp of male donors, indicating that this extract also possesses interesting 5αR-independent properties to modulate HF growth and health.

Saw palmetto extracts have been widely used in hair care products, and as an adjunct or alternative to 5αR-inhibiting drugs, such as finasteride or dutasteride, for preventing hair loss [24, 63–66]. Indeed, in a comparative two-year study, oral monotherapy with *Serenoa repens* dry extract increased hair growth in 38% of patients clinically diagnosed with mild-to-moderate MPHL, compared to 68% of patients in the finasteride group [26].

The efficiency of different saw palmetto extracts greatly varies and depends on several factors, including plant source and harvesting conditions, purity and concentration, bioactive compound profile and extraction method [28, 67]. USPlus® DERM is produced via an advanced proprietary technique known as DeepExtract™, which employs ultrahigh-pressure supercritical CO_2. This method achieves efficacy comparable to solvent extractions such as hexane-based processes, yet it eliminates the potential for residual solvent contamination. This allows the extract to possess the highest amounts and availability of four key proprietary FFAs essential to hair growth, compared to other saw palmetto extracts.

The data reported here, obtained from in vitro experiments in HFDPCs, indicate that USPlus® DERM has a stronger 5αR inhibitory potential than other saw palmetto extracts, reaching approximately 90% of finasteride's inhibitory potential. This suggests that the differing, rich content of free FFAs significantly enhances the nutraceuticals' ability to inhibit 5αR. This may be attributed especially to the higher content of oleic, linoleic and lauric acid [68, 69]. Interestingly, FFAs can form microemulsions and vesicles, which enhance their and the carrier's diffusion through the skin barriers, leading to better therapeutic outcomes [70, 71]. This could enhance the potential to penetrate deeper into the HF during organ culture, reaching the DP more efficiently, where 5αR is expressed [72].

Saw palmetto extracts have been well-documented for their strong 5αR inhibitory effects, making them the primary active ingredient in many commercial nutraceuticals [73]. However, the rich content of FFAs in USPlus® DERM also promises to have additive significant benefits for HF function. Indeed, the cortex and medulla of hair shafts are rich in FFAs, which are essential for hair strength and quality [74]. Furthermore, FFAs have been shown to possess also biological functions [75]. For instance, myristoleic acid has been shown to enhance Wnt signalling in HFDPCs [33]. In mice skin and prostate cancer cells,

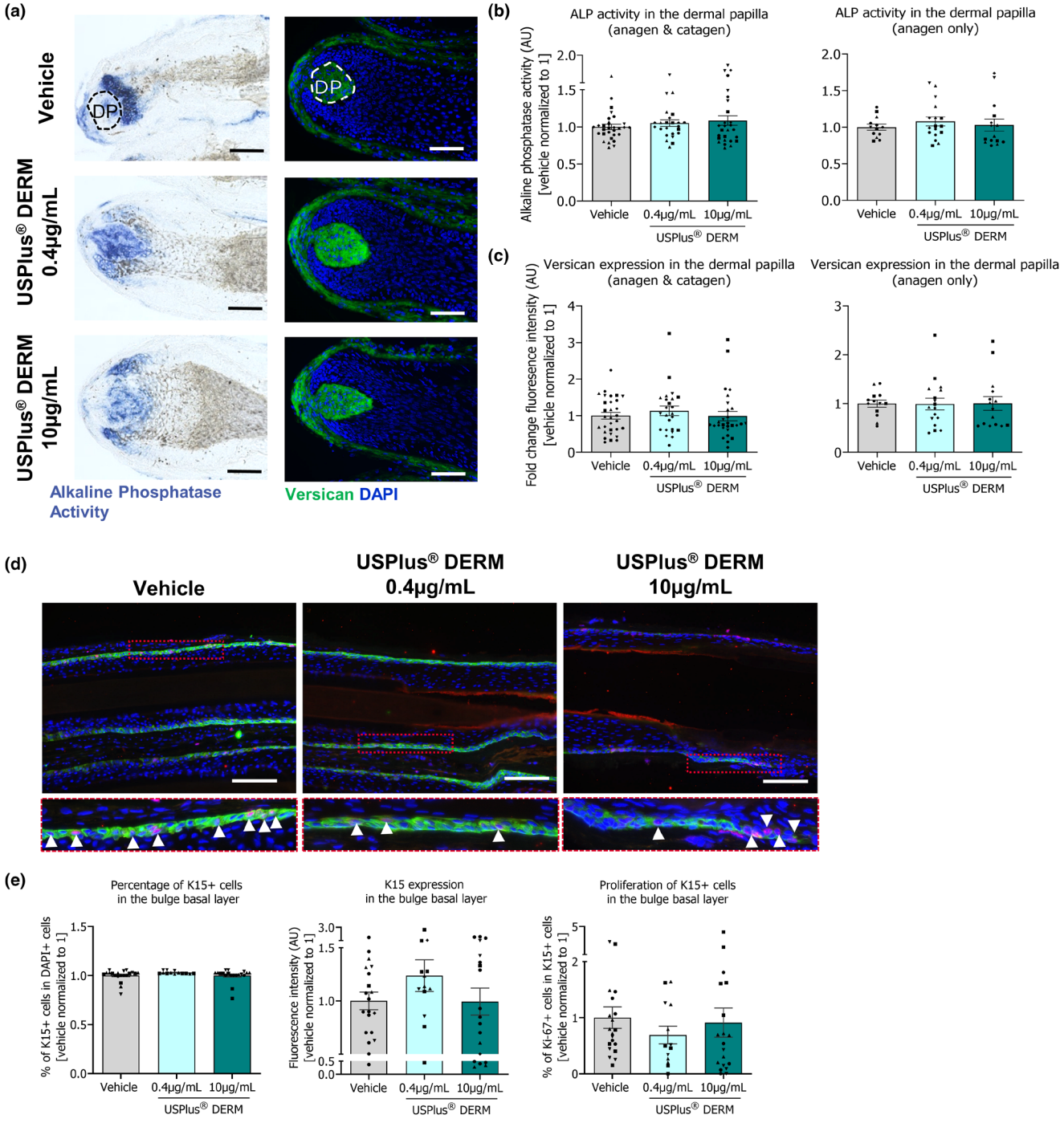

FIGURE 3 USPlus® DERM does not affect DP inductivity, but reinforces the eHFSC stem cell niche in healthy human HFs ex vivo. Hair follicles (HFs) were treated with vehicle control or USPlus® DERM (0.4 or 10 µg/mL) for 5–9 days ex vivo. (a) Representative images of alkaline phosphatase activity and versican expression. (b) Quantification of alkaline phosphatase activity, $n = 24–30$ HFs/ group (all HFs), 13–18 HFs/ group (only anagen) from five to six healthy donors. (c) Quantification of versican expression, $n = 24–30$ HFs/ group (all HFs), 13–18 HFs/ group (only anagen) from five to six healthy donors. (d) Quantification of the percentage of $K15^+$ epithelial hair follicle stem cells (eHFSCs) in the bulge, $K15^+$ expression, and proliferating eHFSCs ($K15^+$/Ki-67^+ cells), $n = 13–20$ HFs/ group from three to four healthy donors. All data are presented as mean ± SEM. Dots of different shape indicate different donors. D'Agostino & Pearson omnibus normality test, no Gaussian distribution; Kruskal–Wallis test, with Dunn's multiple comparison test—vehicle fixed, or Mann–Whitney test—vehicle fixed, data were not siginifcant (ns), Scale bars = 100 µm. DP: Dermal papilla.

Serenoa repens promotes the mitochondrial signalling pathway [76, 77]. In addition, human anagen HFs depend on substantial energy, primarily sourced from aerobic glycolysis, which can utilize FFAs as a metabolic resource [78]. Furthermore, the low molecular weight and straight-chained structure of lauric acid allows it to penetrate the

hair shaft [79], where it could help to stabilize and moisturize the hair. This suggests a great potential of USPlus® DERM for improving hair shaft quality and further underlines its suitability for inclusion in hair care products. Despite the potential of the FFAs present in USPlus® DERM to beneficially affect hair growth and quality, very few studies have explored the 5αR-independent activity of saw palmetto extracts, and consequently the FFAs they contain, on HF biology.

Our pilot encouraging results demonstrate that USPlus® DERM effectively reduced premature catagen conversion under testosterone-free conditions in 'androgen-independent' healthy full-length HFs obtained from the occipital scalp of male donors. These findings suggest a potential anagen-prolonging effect of USPlus® DERM also in vivo, irrespective of its 5αR inhibitory capabilities. Thus, the beneficial effects observed in vivo after USPlus® DERM topical application in female and male AGA patients may also be the result of 5αR-independent mechanisms [29]. While it is conceivable that these properties may be attributed also to other saw palmetto extracts, further studies are warranted to clarify whether changes in the specific content, availability and amount of FFAs, obtained through the DeepExtract® proprietary, ultrahigh-pressure carbon dioxide extraction technology, may alter efficacy in this regard. Particularly, the interesting observation that 0.4 μg/mL USPlus® DERM was more effective in prolonging anagen ex vivo than 10 μg/mL also supports the assumption that a defined composition and balanced concentrations of FFAs are of utmost importance to achieve the most efficient beneficial effects of saw palmetto extracts on HF function. Indeed, an excessive amount of FFA, as likely present in 10 vs. 0.4 μg/mL, has no additional hair growth-promoting effect or could even be potentially detrimental, since high concentrations of FFAs can lead to lipotoxicity, oxidative stress, inflammation, apoptosis and mitochondrial dysfunction [80–83]. Hence, the unique formulation and extraction method of saw palmetto extracts seem to be particularly significant in maximizing their therapeutic benefits.

Unlike in epidermal keratinocytes, where unsaturated FFAs interfere with their differentiation [84], USPlus® DERM did not seem to directly alter hair matrix keratinocyte proliferation in our experiments. Although it was slightly enhanced in the presence of 0.4 μg/mL USPlus® DERM when all HFs were analysed, this finding reflects the higher number of anagen HFs at the end of the culture rather than a direct effect on hair matrix keratinocytes. Furthermore, Serenoa repens has been linked to the activation of intrinsic apoptotic pathways [76]. However, in this study, no significant change was observed in the percentage of apoptotic hair matrix keratinocytes, indicating that USPlus® DERM may affect different pathways rather than cell survival in HFs ex vivo.

It was also shown that FFAs enhance inductivity in DP cells in vitro [36]. However, USPlus® DERM did not affect versican expression or alkaline phosphatase activity, suggesting that increased DP inductivity is not the mechanism underlying the anagen-prolonging effect of USPlus® DERM, at least under testosterone-free conditions.

Previous studies in benign prostatic hyperplasia highlight the potential of *Serenoa repens* to protect human prostate cells from oxidative stress in vitro and in clinical trials [85–88]. In line, linoleic and oleic acid have been indicative of having antioxidant properties in bovine mammary endothelial cells and *C. elegans* [89, 90] Oxidative stress is suggested to contribute also to hair loss observed in AGA, ageing and telogen effluvium [91, 92]. Thus, USPlus® DERM may provide protection against the accumulation of reactive oxygen species. Our findings indicate that USPlus® DERM at lower concentrations prevents melanin clumping caused by abnormal melanin transfer from melanocytes to keratinocytes in the hair matrix [52]. In HF organ cultures, melanin clumping results from stress conditions and is linked to cytotoxicity [52]. Given that melanocytes are particularly susceptible to oxidative stress, which is common during organ culture, especially after HF microdissection [44], the reduced melanin clumping suggests that USPlus® DERM has the potential to protect HFs from oxidative stress.

USPlus® DERM exhibited also a trend toward increased K15 expression while simultaneously reducing proliferation levels of the eHFSCs. This suggests a potential regulatory role of lower concentrations of USPlus® DERM in supporting the equilibrium and quiescence of stem cell activity in the HF bulge [57]. This is in line with previous studies indicating that linoleic and oleic acids inhibited bone marrow-derived mesenchymal stem cell proliferation [93].

Besides these properties, it is expected that USPlus® DERM may regulate other signalling pathways in the HF involved in hair growth. Indeed, FFAs have been linked to adipocyte function and the enhancement of hair growth through the regulation of bone morphogenetic protein signalling and the secretion of growth factors [31]. Yet, additional follow-up investigation will be required to obtain insights into the mechanisms underlying the 5αR-independent hair growth-promoting effects of USPlus® DERM. In particular, future research should be conducted to determine whether the pathways identified for other lipidosterolic extracts of Serenoa repens (LSESr) and fatty acids, such as modulation of inflammatory, anti-oxidant, as well as Wnt signalling, are relevant to explaining the 5αR-independent hair growth properties of USPlus® DERM.

In addition, as a direct comparison of USPlus® DERM and finasteride in the HF model used in the study

(androgen-independent occipital scalp HF organ culture) was not feasible—given that finasteride has no measurable effect on these follicles—future studies should assess both compounds in androgen-sensitive frontal/vertex HFs from AGA patients. While our in vitro data indicate that USPlusDERM® exhibits less 5α-R dependent activity than finasteride, it may still offer therapeutic value via additional 5α-R independent mechanisms, particularly considering the effects observed outside the dermal papilla.

CONCLUSION

In conclusion, USPlus® DERM has significantly enhanced efficacy in inhibiting 5αR, outstanding other standardized saw palmetto extract formulations. Also, USPlus® DERM significantly maintains HF growth under androgen-independent conditions ex vivo, indicating that the anagen-maintaining effect of topically applied USPlus® DERM serum, observed in AGA patients in vivo [29], may not be entirely dependent on 5αR inhibition. Our data suggest that the higher richness of FFA content in USPlus® DERM might additionally support HF health and quality. It remains to be investigated whether other saw palmetto extracts can achieve similar effects, or whether the unique FFA availability and/or composition and distinct benefits of USPlus® DERM on HF function observed here can be specifically attributed to the DeepExtract® method. Nevertheless, our findings suggest USPlus® DERM as a promising strategy for not only AGA but also other androgen-independent hair loss, such as some forms of female pattern hair loss [5], telogen effluvium or even in ageing-associated hair loss [91, 92].

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CONFLICT OF INTEREST STATEMENT

ALR, DB, SA, FJ, JE and MB are employees or consultants of QIMA Monasterium GmbH, a CRO providing preclinical and clinical research services. VL is an employee of Valensa International, Eustis, FL, USA.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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