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Serenoa repens extracts promote hair regeneration and repair of hair loss mouse models by activating TGF-β and mitochondrial signaling pathway

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Abstract. – OBJECTIVE: Plenty of plant extracts have been used for treating hair loss. This study aims to investigate the effects of liposterolic extracts of Serenoa repens (LSESr) on hair cell growth and regeneration of hair, and clarify the associated mechanisms.

MATERIALS AND METHODS: Human keratinocyte cells (HACAT) were cultured, incubated with dihydrotestosterone (DHT) and treated with LSESr. Cell viability was examined by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Hair loss C57BL/6 mouse model was established by inducing with DHT. Hair growth, density, and thickness were evaluated. Back skin samples were collected and stained with hematoxylin and eosin (HE) assay. B-cell lymphoma-2 (Bcl-2), Bcl-2 associated protein X (Bax), cleaved caspase 3 and transforming growth factor β2 (TGF-β2) were examined using Western blot assay.

RESULTS: LSESr treatment significantly increased HACAT cell viabilities compared to DHT-only treated cells ($p<0.05$). LSESr treatment post injection of DHT significantly converted skin color from pink to gray and increased hair density, weight and thickness compared to DHT-only treated mice ($p<0.05$). LSESr treatment significantly triggered follicle growth and decreased inflammatory response. LSESr treatment significantly decreased TGF-β2 and cleaved caspase 3 expression of hair loss mouse models compared to that of DHT treated mice ($p<0.05$). LSESr treatment significantly enhanced Bcl-2 expression and reduced Bax expression compared to that of DHT treated mice ($p<0.05$). Meanwhile, effects of LSESr were substantial even achieving to the potential of finasteride.

CONCLUSIONS: LSESr promoted the hair regeneration and repair of hair loss mouse models by activating TGF-β signaling and mitochondrial signaling pathway.

Key Words:

Serenoa repens extracts, Hair loss, Hair regeneration, Dehydrotestosterone.

Introduction

In animal or human body, hair is the fastest growing tissue[1,2]. The hair follicle undergoing the repetitive regenerative cycles consists of three stages, including anagen stage, catagen stage and telogen stage[3]. Actually, during the processes of hair loss, the growth of hair usually illustrates multiple abnormalities, such as premature catagen, curtailed anagen[4]. Meanwhile, the hair follicles exhibit the changes to be the terminal hair follicles[5].

In clinical, the androgenetic alopecia (AGA) is the most frequently occurred category of hair loss in men, which affects even 50% males in the whole world[6]. AGA is defined as the hair loss caused by the inherent factors and the secretion of the androgens, such as testosterone, dihydrotestosterone (DHT)[7]. Nowadays, the exact genetic factors participating in the hair loss have not been fully clarified, but a few candidate genes associated with the hair loss are discovered, such as fibroblast growth factor, epidermal growth factor, lymphoid-enhancer factor[8]. Clinically, the most commonly used approaches for hair loss therapy are finasteride (Fin), topical minoxidil and the transplantation of hair[9]. However, the above methods are not effective for all of the hair loss patients due to the adherence of patients, side-effects of drugs, limited hair donors[10]. Due to the above limitations and clinical requirements, the traditional Chinese herb medicine has been considered as a promising and tendency for the hair loss treatment.

To date, a plenty of plant extracts have been used for treating hair loss in China, United State and Europe. The most common plant extracts are extracted from the *Serenoa repens* tree and it's fruit[11]. The *Serenoa repens* extracts play many

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biological roles, including anti-inflammation, anti-androgen, anti-proliferation effects[12]. The previous study[13] reported that the inhibitor of 5-adrogen receptor (5-AR) and anti-androgen drugs are effective to treat the hair loss. The liposterolic extracts of *Serenoa repens* (LSESr) exhibit higher efficacy for inhibiting 5-AR *in vitro* assay compared to the Fin, which is commonly used in clinical[13].

As mentioned above, the *Serenoa repens* extracts could treat the hair loss by inhibiting 5-AR expression; however, its effects on cell growth and hair regeneration or repair *in vivo* animal models are not clarified. Therefore, this study aims to investigate the effects of LSESr on growth of hair cell HACAT cells *in vitro* and regeneration of hair *in vivo*, clarifing the associated mechanisms. Also, the findings obtained by application of LSESr were compared with that of Fin.

Materials and Methods

Cell Culture

The human keratinocyte cells (HACAT) were purchased from Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China). HACAT cells were cultured in the low-glucose Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NJ, USA), supplementing with the 10% fetal bovine serum (FBS, Gibco, Grand Island, NJ, USA), 100 μg/ml streptomycin (Beyotime Biotech., Shanghai, China) and 100 U/ml penicillin (Biyotime Biotech., Shanghai, China). The HACAT cells were cultured at 37°C and 5% CO_2, and sub-cultured growing to 80% confluency at the secondary day on 6-well plates (Corning Costar, Corning, NY, USA).

Cell Change and Trial Grouping

When the density of HACAT cells achieved to 5×10^3 cells/ml, the culture was put into the 6-well or 96-well plates (Corning Costar, Corning, NY, USA) for 24 h. The LSESr, DHT and Fin were prepared by dissolving into the dimethyl sulfoxide (DMSO, Amresco Inc., Solon, OH, USA). The above reagents were dissolved into serum free media. For the DHT-induced cells, the HACAT was treated with the 0.3 μg/ml DHT (Sigma-Aldrich, St. Louis, MI, USA). Then, the DHT-induced HACAT cells were divided into DHT group, DHT+Fin group (by treating with 0.08 μg/ml Fin, Sigma-Aldrich, St. Louis, MO, USA) and DHT+LSESr group (by treating with 1, 5, 25, 100 and 200 μg/ml LSESr, respectively). The LSESr was purchased from Yongyuan Biotech. Co., Ltd. (Xi'an, China). Meanwhile, the HACAT cells un-treated with reagents were assigned as Blank group.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) Assay

HACAT cells were treated as the above processes. About 24 h, 48 h and 72 h after the DHT treatments, the HACAT cells were cultured in 96-well plates and treated with MTT (Sigma-Aldrich, St. Louis, MO, USA) at final concentration of 5 mg/ml for 4 h. Then, the MTT induced formazan was dissolved by using the dimethyl sulfoxide (DMSO) solution. The absorbance of the 96-well plates was examined by using the enzyme linked immunosorbent assay (ELISA) reader (Thermo Scientific Pierce, Waltham, MA, USA) at 570 nm.

Establishment of DHT-Induced Hair Loss Mouse Model and Trial Grouping

A total of 32 C57BL/6 specific-pathogenic-free (SPF) mice (Beijing HFK Biosci. Co. Ltd., Beijing, China) aging from 6 to 8 weeks and weighting from 18 to 22 g, were employed in this study. All of the mice were denuded at a uniform time point by utilizing animal clippers (Shanghai Medical Instruments Group, Ltd. Corp., Shanghai, China) and hair removal cream (Ping Yu Maya Biotech. Co. Ltd., Shanghai, China). This study was approved by the Ethical Committee of Jiangyin hospital affiliated to Nanjing University of TCM (Jiangyin, China).

For DHT-induced hair loss mouse model (24 mice), the 0.5 ml of DHT was multiply-points injected into the neck region for each mouse for 5 weeks. Then, the DHT-induced hair loss mouse models were divided into DHT group (n=8), DHT+Fin group (n=8, by intragastrically administrating with 0.01% Fin solution daily for 5 weeks) and DHT+LSESr group (n=8, by intragastrically administrating with 50% LSESr solution daily for 5 weeks). Meanwhile, the other 8 mice untreated with any drugs were assigned as Blank group.

Hair Density Evaluation

The digital graphs were captured by using camera and analyzed by using the Image J software (version: 1.45s, National Institutes of Health, Bethesda, MD, USA). The hair density and thickness were evaluated and analyzed by selecting 5 independent areas of 25 mm^2 size in each mouse by using the Aram diagnosis scope (Aram Human Vision System, Sungnam, Korea).

Back Skin Samples Collection and Hematoxylin and Eosin (HE) Staining

The mice were anesthetized by utilizing the 7% chloral hydrate at the concentration of 0.5 ml/100 g weight body on 35 days. The dorsal skins were incubated with 4% formaldehyde (Beyotime Biotech. Shanghai, China) in phosphate-buffered solution (PBS, Biyotime Biotech, Shanghai, China). Then, the dorsal skins treated by paraffin block embedding.

In this study the histology was visualized by employing the HE staining according to the previous study described[14]. The stained skin tissues were observed and captured by using the optical microscope (Mode: BX51, Olympus, Tokyo, Japan). The HE stained slides were captured by utilizing the digital microscope (Mode: DSX110, Olympus, Tokyo, Japan) and the graphs were cropped to a fixed area of 300 pixel wide. Finally, the digital graphs were collected from the representative areas with the magnification of 400 ×.

Western Blot Assay

The expression of proteins in skin tissues was detected by using the Western blot assay. The skin tissues were collected according to the above methods described. The skin tissues were lysed by utilizing the tissue homogenate machine (Scientz Bio. Tech. Inc., Ningbo, China) and tissue total protein lysis buffer (Biyotime Biotech. Shanghai, China) according to the instructions of manufacturers. The protein lysates were separated by employing 15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, Sangon Biotech. Co. Ltd., Shanghai, China), and electrotransferred onto polyvinylidene fluoride (PVDF, Bio-Rad Laboratories, Hercules, CA, USA). The PVDF membranes were blocked by using 5% defatted milk (BD Biosciences, San Jose, CA, USA) in PBS containing 0.05% Tween-20 solution and pH 7.5), and incubating with the rabbit anti-mouse transforming growth factor β2 (TGF-β2) polyclonal antibody (1: 2000; Catalogue No. ab113670, Abcam Biotech., Cambridge, MA, USA), rabbit anti-mouse cleaved caspase 3 polyclonal antibody (1: 2000; Catalogue No. ab13847, Abcam Biotech., Cambridge, MA, USA), rabbit anti-mouse B-cell lymphoma-2 (Bcl-2) polyclonal antibody (1: 2000, Catalogue No. ab59348, Abcam Biotech., Cambridge, MA, USA), rabbit anti-mouse Bcl-2 associated protein X (Bax) polyclonal antibody (1: 2000, Catalogue No. ab182733) and rabbit anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1: 3000, Catalogue No. ab181603) at 4°C overnight. PVDF membranes were incubated with 1: 3000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Catalogue No. ab6721, Abcam Biotech., Cambridge, MA, USA). The Western blot bands were visualized with enhanced chemiluminescence kit (ECL, Pierce, Rockford, IL, USA). The Western blot bands were captured and analyzed by using UVP gel scanning system (Mode: GDS8000, UVP, Sacramento, CA, USA).

Statistical Analysis

Data were illustrated as mean ± standard deviation (SD) and were analyzed by using SPSS software 20.0 (SPSS Inc., Chicago, IL, USA). The data were obtained from at least six independent tests or experiments. Student's *t*-test was utilized to compare differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. A statistical significance was defined when $p<0.05$.

Results

LSESr Treatment Increased HACAT Cell Viabilities

In order to investigate the effects of LSESr on HACAT cell viabilities, the MTT assay was performed. The results showed that DHT significantly decreased the cell viability compared to the Blank group (Figure 1A, $p<0.05$) at 24 h after culture of cells. The Fin treatment significantly rescued the DHT decreased cell viability compared to the DHT group (Figure 1A, $p<0.05$). Moreover, when the DHT-induced cell treating with 25, 100 and 200 μg/ml, the cell viabilities were significantly increased compared to DHT group (Figure 1A, $p<0.05$). Moreover, LSESr treatment also significantly increased the HACAT cell viabilities compared to DHT group at 48 h (Figure 2B) and 72 h (Figure 2C) post culture, respectively ($p<0.05$).

LSESr Treatment Improved Hair Growth of DHT-Induced Hair Loss Mouse Model

The effects of LSESr treatment on hair growth of DHT-induced hair loss mouse model were evaluated in this study. The results indicated that LSESr treatment after the injection of DHT significantly converted the skin color from the pink to the gray initiating at the 1st week; the hair growth started at the 3rd week and was covered at the 5th week (Figure 2A). The hair growth un-

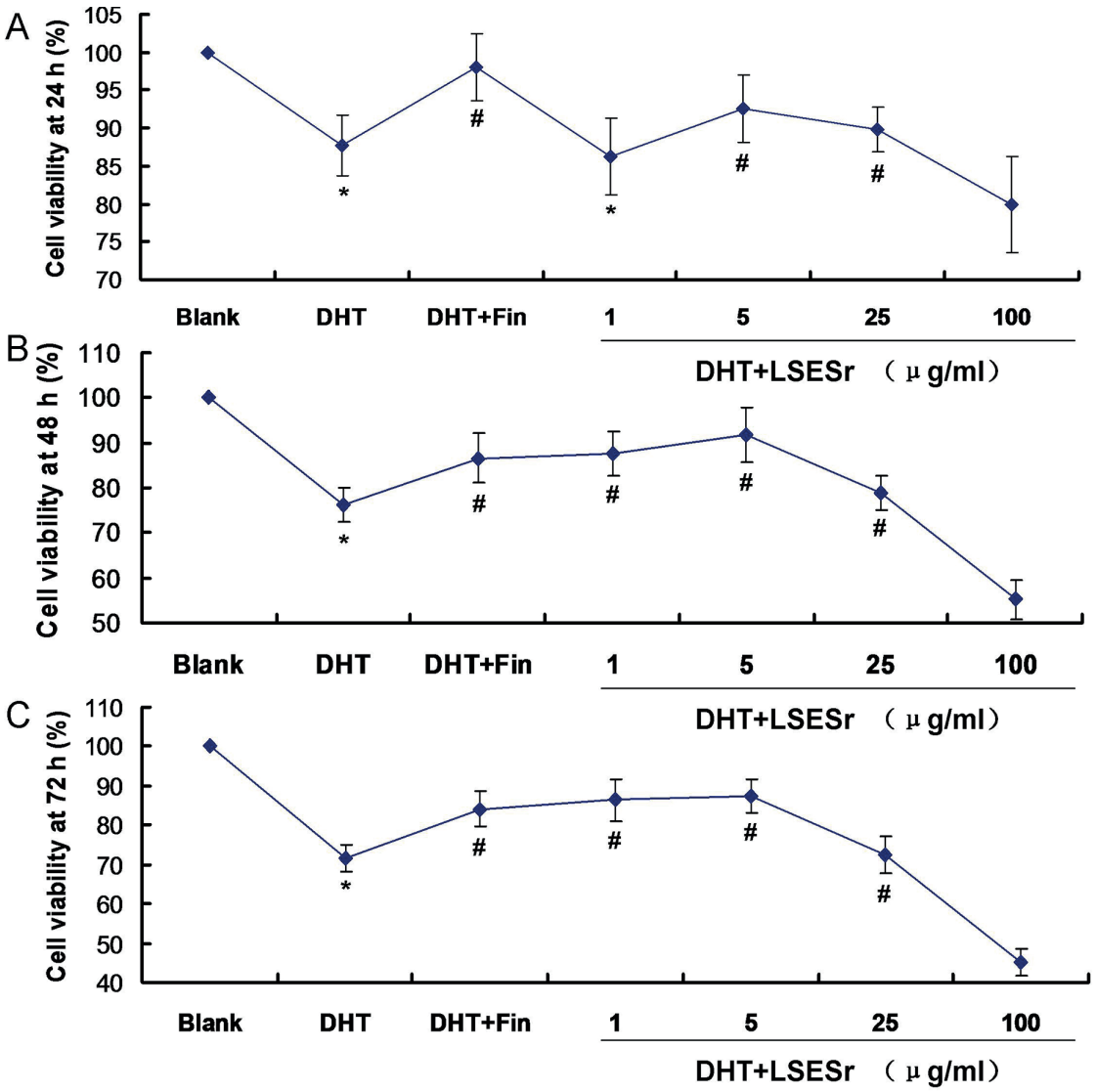

Figure 1. Cell viabilities of HACAT cells undergoing LSESr treatment. ***A,*** Cell viabilities observation at 24 h post LSESr treatment. ***B,*** Cell viabilities observation at 48 h post LSESr treatment. ***C,*** Cell viabilities observation at 72 h post LSESr treatment. $^{*}p<0.05$ *vs.* Blank group, $^{\#}p<0.05$ *vs.* DHT group. LSESr: *Serenoa repens* extracts, HACAT: Human keratinocyte cells, Fin: finasteride.

dergoing LSESr treatment was significantly better compared to that in the DHT-induced mouse model receiving no LSESr (DHT group), and even achieved to the conditions of Fin treatment group (Figure 2B). Meanwhile, the hair weight in DHT group was significantly decreased compared to Blank group (Figure 1B, $p<0.01$). The LSESr treatment after the injection of DHT significantly increased hair weight compared to DHT group (Figure 2B, $p<0.01$); however, its levels were significantly lower compared to the Blank group (Figure 2B, $p<0.05$).

LSESr Treatment Triggered Follicle Growth and Decreased Inflammatory Response

All of the mice were sacrificed at 35th day after the depilation and the skin tissues were isolated and stained by using HE staining method for the histological analysis. The results showed that there were longer and larger follicles in the LSESr treated mice compared to the DHT-only treated mice or the Fin-treated mice (Figure 3). The samples in LSESr treated mice exhibited typical catagen-like morphology of follicle (Figure

3). Furthermore, the DHT induced plenty of inflammatory cells in the tissues of hair loss mouse model, which were reversed by treating with the LSESr and Fin, respectively (Figure 3).

LSESr Treatment Decreased TGF–β2 and Cleaved Caspase 3 Expression

The cell proliferation and apoptosis biomarkers TGF-β2 and cleaved caspase 3 expressions were examined by using Western blot assay (Figure 4A). The results showed that the expression of TGF-β2 is significantly lower compared to the DHT group in the groups treating with the LSESr (Figure 4B, $p<0.05$) and Fin (Figure 4C, $p<0.01$), respectively. Additionally, the cleaved caspase 3 was also significantly decreased in LSESr treatment group (Figure 4B, $p<0.05$) and Fin treatment group (Figure 4C, $p<0.01$) compared to the DHT group. The effects of LSESr were substantial even achieving to the potential of the Fin (Figure 4).

LSESr Treatment Inhibited Apoptosis by Activating Mitochondria Signaling Pathway

In order to clarify the reasons for the LSESr triggered hair growth, the key factors of mitochondria signaling pathway were evaluated by Western blot (Figure 5A). The results showed that the Bcl-2 levels (Figure 5B) were significantly decreased and Bax levels (Figure 5C) were si-

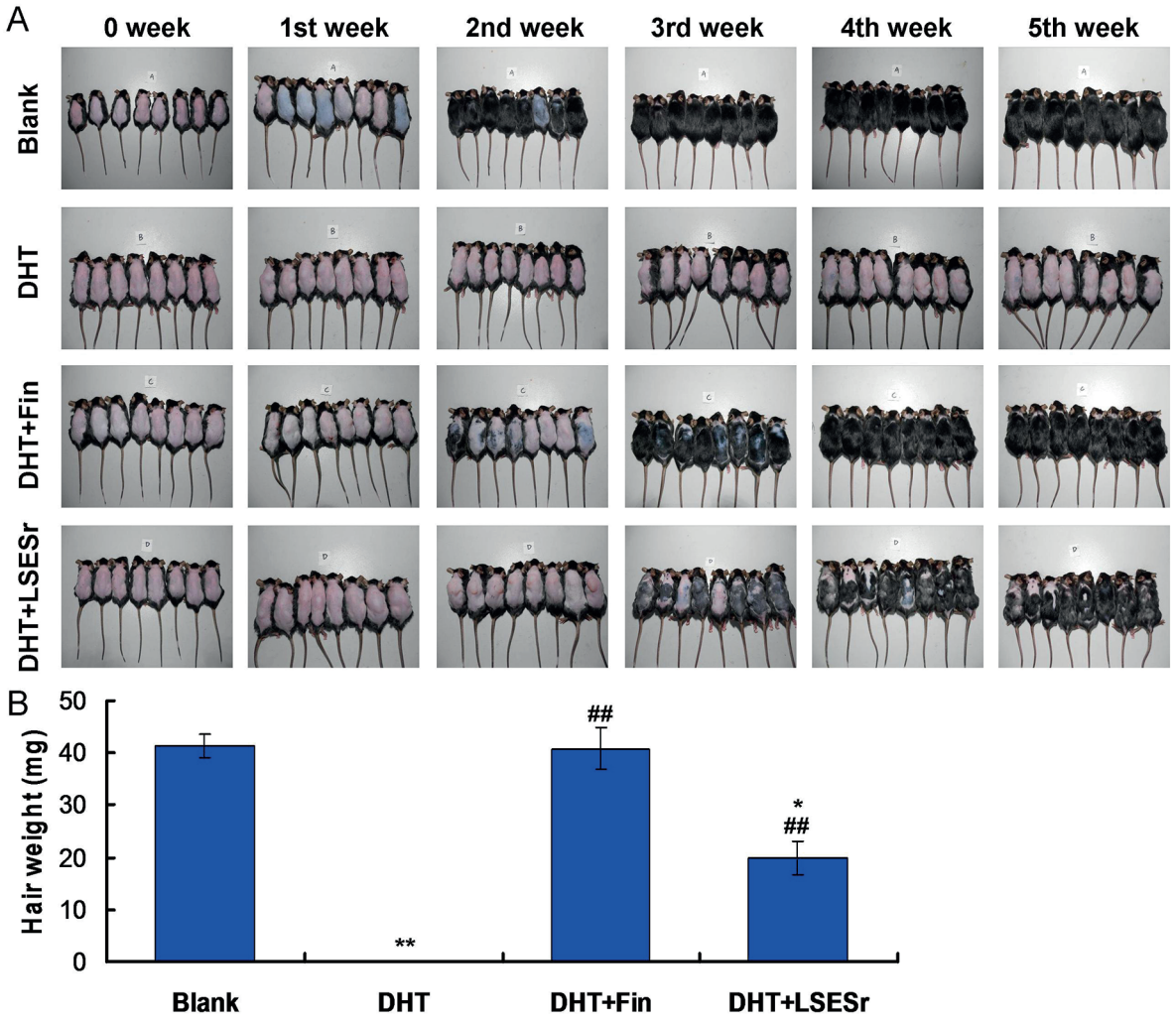

Figure 2. The effects of LSESr and Finasteride on the hair growth in DHT-induced hair loss mouse model. ***A,*** Graphs for the hair growth of hair loss mouse models from 0 week to 5 weeks undergoing LSESr treatment. ***B,*** Statistical analysis for the hair weight of mouse models. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Blank group, $^{\#\#}p<0.01$ *vs.* DHT group. LSESr: *Serenoa repens* extracts, DHT: dihydrotestosterone, Fin: finasteride.

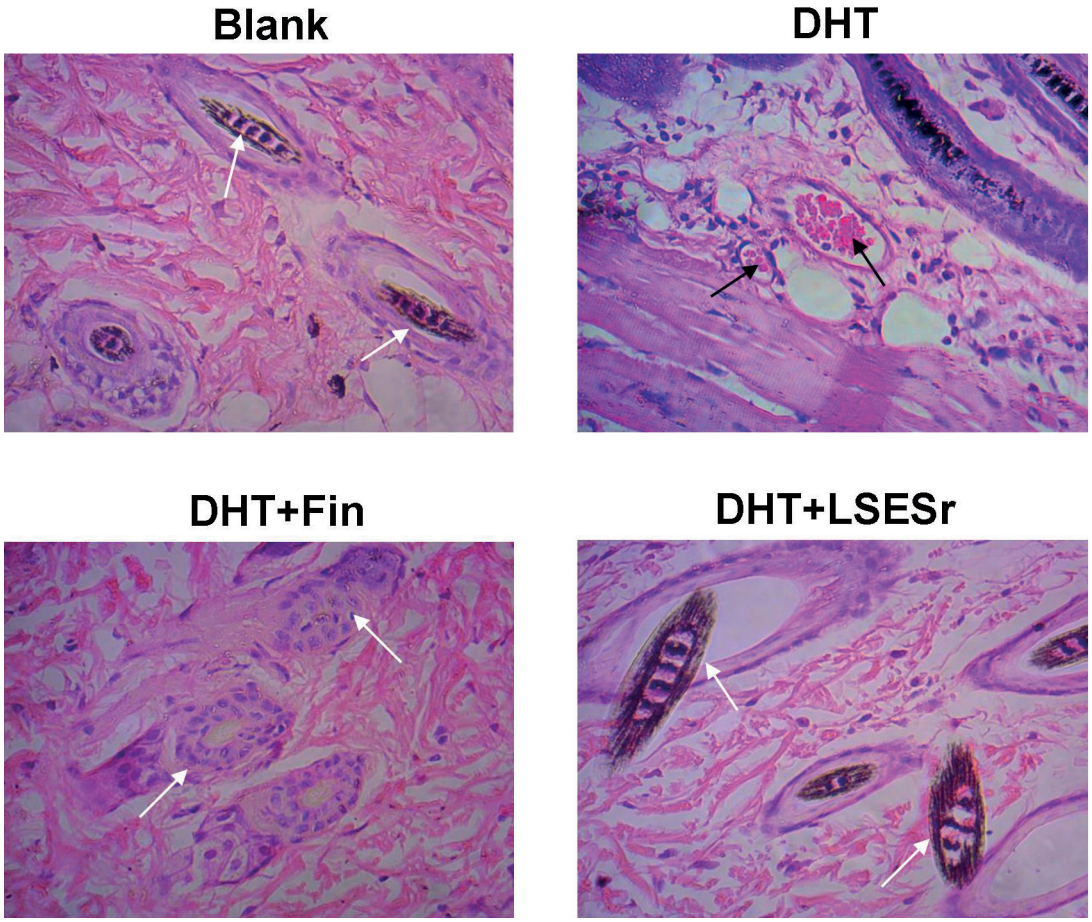

Figure 3. Comparison for the histological graphs of hair follicles and inflammatory cells. White arrows represent the hair follicles. Black arrows represent the inflammatory cells. LSESr: *Serenoa repens* extracts, DHT: dihydrotestosterone, Fin: finasteride (400 ×).

gnificantly increased in DHT group compared to Blank group ($p<0.05$). The LSESr treatment significantly increased the Bcl-2 levels compared to the DHT group (Figure 5B, $p<0.05$), which were even equal to the levels of DHT+Fin group. Meanwhile, the LSESr treatment also significantly decreased the Bax levels compared to the DHT group (Figure 5C, $p<0.05$), which even deduced to the levels of DHT+Fin group.

Discussion

In the present study, we exhibited that the LSESr treatment could increase the proliferation of HACAT cells and could promote the hair regeneration and repair in the DHT-induce hair loss mouse models. In our experiments, the LSESr turned to be more promising or potent in enhancing hair cell growth and improving hair regeneration.

The plant extracts comprise a plenty of chemical factors that play the effects on the cellular physiology, and have potential in targeting several diseases[15,16]. Particularly, the plant-derived or dependent treatment has been proven to be effective in treating the inflammatory skin diseases, such as the hair loss, atopic dermatitis[17]. In this work, we discovered that treating HACAT cells with LSESr resulted in a significant increase of HACAT cell viabilities compared to DHT-induced HACAT cells at 24 h, 48 h and 72 h, respectively. The previous research[18] also reported that LSESr induced nothing of negative effects on the cell viabilities, consistently with our findings. Therefore, these results suggest that the LSESr treatment could promote the HACAT cell growth, and may improve the hair growth *in vivo* levels.

Due to the well-known characteristics of the C57BL/6 mouse, it has been extensively applied in establishing the hair growth model[19]. The DHT

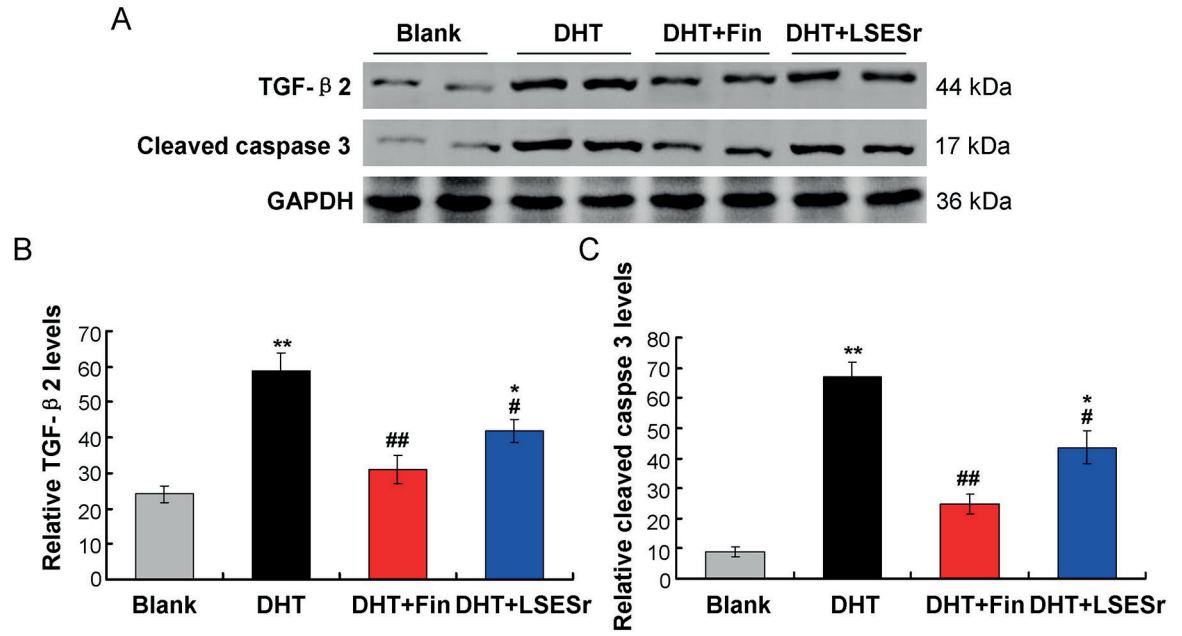

Figure 4. Evaluation for the TGF-β2 and cleaved caspase 3 expression in skins of hair loss mouse models. ***A***, Western blot images for the TGF-β2 and cleaved caspase 3 expression. ***B,*** Statistical analysis for the TGF-β2 and cleaved caspase 3 expression. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Blank group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* DHT group. LSESr: *Serenoa repens* extracts, DHT: dihydrotestosterone, Fin: finasteride, TGF-β2: transforming growth factor β2.

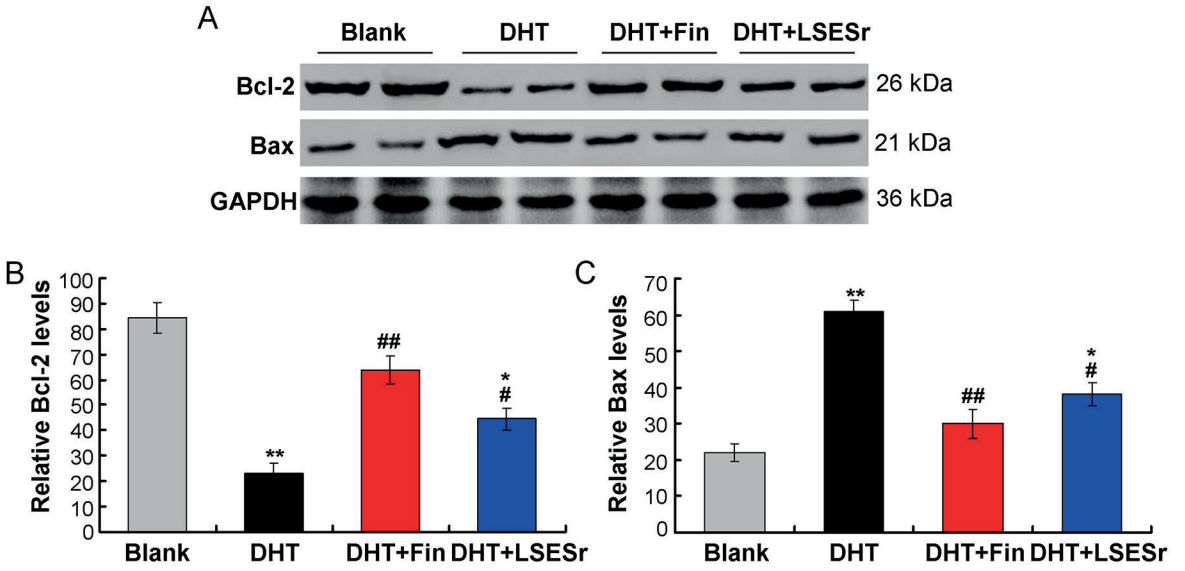

Figure 5. Observation for the Bcl-2 and Bax expression in skins of hair loss mouse models. ***A,*** Western blot images for the Bcl-2 and Bax expression. ***B,*** Statistical analysis for the Bcl-2 and Bax expression. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Blank group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* DHT group. LSESr: *Serenoa repens* extracts, DHT: dihydrotestosterone, Fin: finasteride, Bcl-2: B-cell lymphoma-2, Bax: Bcl-2 associated protein X.

treatment induced the hair cell growth inhibition and entering into telogen and catagen stage[20]. In the *in vivo* investigations, the hair density, hair coverage and hair thickness post the LSESr administration were evaluated. The results showed that the hair growth undergoing LSESr treatment was significantly better than that in DHT-induced mouse model receiving no LSESr. Meanwhile,

LSESr treatment post injection of DHT significantly increased hair weight compared to DHT group. These results suggest that the LSESr was effective in preventing the hair loss, the effects of which even comparable to the Fin. Our results are consistent with Shin et al[4] work illustrating the effects of LSESr on hair loss. Moreover, the LSESr may also inhibit the hair cell apoptosis and promote the hair cell growth. Therefore, we discussed the mechanism for the protective effects of LSESr on hair cells.

The HE staining results showed that more longer and larger follicles were appeared in skins of LSESr treated mice; however, the follicles were even disappeared in skins of DHT-only treated mice. Also, the LSESr significantly inhibited the DHT induced inflammatory cells accumulating in the tissues of hair loss mouse model. These findings were critical for the hair loss in the mouse model, consistently with the previous published study[4].

The previous reports[21,22] proved that the molecules involving in apoptotic processes include anti-apoptotic protein (Bcl-2), pro-apoptotic protein (Bax), TGF-β2 and cleaved caspase 3, all of which considered to be critical factors in hair loss. Especially, the TGF-β2 is the most critical factor for entering to catagen during the process of the hair cells, and affects the apoptosis associated factors, such cleaved caspase 3, Bcl-2 and Bax[23-25]. The results indicated that the induction of TGF-β2, cleaved caspase 3, Bax, and reduction of Bcl-2, by treating with DHT were prevented by the LSESr. Meanwhile, the effects of LSESr were consistently similar to that of Fin. All of these results suggest that the mitochondrial signaling pathway was involving in the protective roles of LSESr on the hair loss.

Conclusions

In the present report, the molecules involving in the hair cell growth, apoptosis in DHT-induced HACAT cells, were evaluated. The hair growth, hair density, hair thickness and expression of Bcl-2, Bax, cleaved caspase 3 and TGF-β2 in skins of mice, were also examined. We found that the LSESr promoted the hair regeneration and repair of hair loss mouse models by activating the TGF-β signaling and mitochondrial signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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