EFFECT OF OLEANOLIC ACID ON 5A-REDUCTASE ACTIVITY, DPCS PROLIFERATION AND GENE EXPRESSION CORRELATED WITH ANDROGENETIC ALOPECIA IN VITRO

ChunYu Cao[1,2], YuLei Liu[1,3], Qi Ding[4], YuYu Deng[5], QiaoFang Wu[6], YuXuan Xu[1], JingJing Liu[1], WeiXin Fan[1,*]
[1]Department of Dermatovenereology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China - [2]Department of Dermatovenereology, Taizhou People's Hospital, Taizhou, Jiangsu, China - [3]Department of Dermatovenereology, The Affiliated Hospital of Inner Mongolia Medical University, Huhhot, Inner Mongolia, China - [4]Department of Dermatovenereology, The Affiliated Hospital of Nantong University, Nantong, Jiangsu, China - [5]Dermatology Hospital of Jiangxi Province, Nanchang, Jiangxi, China - [6]Department of Dermatovenereology, The Affiliated BenQ Hospital of Nanjing Medical University, Nanjing, Jiangsu, China

ABSTRACT

Objective: *Androgenetic alopecia (AGA) has a serious influence on the appearance and psychology of alopecia patients; however, the current primary treatments have unwanted side effects and transient actions. New drugs for alopecia need to be developed. Oleanolic acid (OA), a natural pentacyclic triterpenoid, has several benefits and medicinal properties. Our previous in vitro study determined that OA showed the strongest effect of promoting hair growth among 55 Chinese herbal medicines; however, data are still scarce, making it difficult to discover the underlying mechanisms responsible for OA promoting hair growth, especially its anti-androgenic effects. This study aims to test the anti-androgenic effects of OA.*

Methods: *Crude enzymes were isolated from SD rat prostate and liver to perform 5α-reductase activity assays. The growth of human Dermal Papilla Cells (DPCs) was measured by the CCK-8 assay. Quantitative Real-Time PCR reactions were performed to determine gene expression changes of DPCs.*

Results: *OA markedly decreased the 5α-reductase activity in a dose-dependent manner. The cell proliferation of cultured DPCs was significantly promoted by OA. Multiple genes associated with the progression of AGA, including SRD5A1, SRD5A2, TGF-β1, TGF-β2, AR, ER, DKK-1, IGF-1 and VEGF, were found to be regulated by OA.*

Conclusion: *OA could directly inhibit 5α-reductase activity, promote DPC proliferation, change DPC gene expression and reduce the impact of DHT on DPC gene expression. OA may promote hair growth through the mechanisms above, and could have potential for use in future treatment against AGA.*

Keywords: *Oleanolic acid, androgenic alopecia, testosterone 5-alpha-reductase, dihydrotestosetrone.*

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Introduction

Alopecia is an increasingly common disorder in both men and women, which is currently highlighted due to its serious influence on the appearance and psychology of alopecia patients. Androgenetic alopecia (AGA), the most common form, shows a rising tendency worldwide; however, preventing this condition is not simple. Even though the demand for alopecia therapy is rising, only two FDA-approved drugs have been authorised for AGA patients: finasteride and minoxidil[1-2]. Finasteride, a type II 5α-reductase inhibitor, can be used in prostatic hypertrophy as well as androgenetic alopecia. Minoxidil was initially used as a drug to treat high blood pressure, but was also found to promote hair growth. In a previous study, minoxidil was shown to have proliferative and anti-apoptotic effects on DPCs. Moreover, it was also found to stimulate hair growth by the up-regulation of vascular endothelial growth factor (VEGF) in DPCs[3]. Dutasteride can inhibit both type 1 and type 2 isoenzymes of 5α reductase[4-5].

Although finasteride, minoxidil and dutasteride treatment have currently achieved certain curative effect, many patients still fail to get significant benefits from these treatments, and some patients report continued hair loss when discontinuing the drug. Because of the unwanted side effects and transient action of finasteride and minoxidil, new targeted drugs for alopecia need to be developed.

A number of pharmacological studies have been performed using various herbal ingredients to test their anti-androgenic effects, and their ability to inhibit 5α-reductase, promote growth factor expression in hair follicles, regulate the endocrine system and enhance microcirculation in recent years. This study therefore aims to test a potential anti-androgenic herb ingredient.

Traditional Chinese medicine has a long history of treating hair loss; one of the most commonly used herbs is Fructus Ligustri Lucidi[6]. Oleanolic acid (OA), a natural pentacyclic triterpenoid, is one of the main bioactive compounds in Fructus Ligustri Lucidi[7], which possess anti-inflammatory, anti-oxidative, antiprotozoal, anti-mutagenic and anticancer properties[8]. Our previous study tested 55 Chinese herbal medicines, and determined that OA from Fructus Ligustri Lucidi showed the strongest effect of promoting hair growth in vitro; specifically, it accelerated the growth of hair shafts and prolonged the growth period[9]. A clinical trial in Japan using swertia extract containing OA to evaluate the hair regrowth effect showed significantly increased numbers of hairs in the 1 cm circle on the scalp after 4 months[10]. However, little research into the anti-androgenic effect of OA has been performed; in particular, the underlying mechanisms of OA promoting hair growth have not been described.

Inhibition of 5α-reductase activity is important in preventing hair loss in AGA[11]. The 5α-reductase enzyme converts testosterone to dihydrotestosetrone (DHT) and is responsible for androgenetic alopecia. DHT, a potent form of testosterone, can cause shortening of the anagen phase, resulting in the miniaturisation of hair follicles. The inhibition of 5α-reductase contributes to the improvement of hair loss in androgenetic alopecia patients[1].

DPCs, fibroblasts of mesenchymal origin, consist of a cluster of specialised fibroblasts that play important roles in the regulation of the hair cycle through the secretion of diffusible proteins, such as insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), VEGF and transforming growth factor-β (TGF-β). Previous reports have shown that the size of the DPCs correlated well with hair growth, and the number of DPCs increased in the growing phase of the hair cycle[12-13].

In this study, we investigated the anti-androgenic effect of OA in vitro, assayed its inhibitory effects on 5α-reductase and promotion effects on DPC proliferation and its influence on multiple genes associated with the progression of AGA, including 5a-reductase I (SRD5A1), 5a-reductase II (SRD5A2), androgen receptor (AR), oestrogen receptor (ER), Dickkopf-1 (DKK-1), insulin-like growth factor-1 (IGF-1), transforming growth factor-beta 1 (TGF-β1), transforming growth factor-beta 2 (TGF-β2) and vascular endothelial growth factor (VEGF).

Methods

Animals

Male SD rats (8-week-old) were purchased from Comparative Medicine Centre of YangZhou University (YangZhou, China) and were provided with standard laboratory conditions.

Assay for 5α-Reductase Activity

Male SD rats (8 weeks) were sacrificed with carbon dioxide (CO_2). The prostate and liver of rats were dissected, freed of their capsules, then washed with saline, and stored at -80°C. Frozen tissues were thawed on ice and procedures were performed at 4°C. The tissues were homogenised with precooled buffer (0.32 M sucrose, 1 mM dithiothreitol (DTT), 0.1mM disodium EDTA, pH 6.8) in 5-6 tissue volumes, then centrifuged at 100,000 g for 60 min. The pellets were recovered, and washed with three tissue volumes of precooled buffer, then centrifuged twice at 400 g at 0°C for 10 min. Finally, the pellets were re-suspended in precooled buffer and stored at -80°C until use. The suspension derived from rat prostate (4.83 mg protein/ml, determined by the Bradford method) and rat liver (14.04 mg protein/ml, determined by the Bradford method) was used as a source of 5α-reductase. Respectively the prostate crude enzyme mainly contained type II 5α-reductase, and the liver crude enzyme mainly contained type I and II 5α-reductase.

5α-reductase activities were assayed according to the method of reference[14]. An independent set of the reaction (n=3) was examined. The 5α-reductase reaction system contained a final volume of 200 μL, and the reaction composition is shown in Table 1. 5α-Reductase activities were analysed as previously described. The reactions were initiated with (0.5, 1, 2, 4, 8, 16 mg/ml) oleanolic acid (Sigma, U.S.A) and were done in 96-well plates. Finasteride 2 nM or Dutasteride 2 nM (Sigma, U.S.A) were used as positive controls and ethanol was used as a negative control. The composition mixture was incubated at 37°C for 30 min, and then the reaction was terminated.

Testosterone had been converted to dihydrotestosetrone (DHT) in the conditions of 5α-reductase, and then a commercial ELISA kit for DHT (Cusabio Technology LLC, WuHan, China) was utilised to detect the content of DHT according to the manufacturer's instructions. First, the DHT standards was diluted into 0, 2.5, 5, 10, 20 and 40ng/ml concentration, and got the absorbance value at 450nm with a Microplate Reader, then drawn the DHT standard curve. When the absorbance values of the 5α-Reductase Reaction system samples and its controls were read, the values were used to obtain the DHT concentration of each experiment group through the standard curve.

The activity of 5α-reductase was expressed as a ratio calculated with the equation:

[DHT/(T+DHT)] × 100.

Composition	Control	Finasteride	Dutasteride	Oleanolic acid
Testosterone	10	10	10	10
5α-reductase extract	50	50	50	50
NADPH	10	10	10	10
Ethanol	10	-	-	-
Finasteride	-	5	-	-
Dutasteride	-		5	-
Oleanolic acid	-		-	10
Buffer	120	125	125	120

Table. 1: The 5α-Reductase Reaction system composition (ul).

Culture and Proliferation Assay of Dermal Papilla Cells

The Human Hair Dermal Papilla Cells (Sciencell, USA) were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 unit/ml, 100 μg/ml, respectively) at 37°C in a humidified atmosphere under 5% CO2. The proliferation of DPCs was evaluated by measuring the metabolic activity using a CCK8 assay. Briefly, 2000 DPCs were seeded into 96-well plates. After 24 h the existing media was aspirated and replaced with 200 μl of fresh media, together with the indicated concentrations of OA and incubated for 1,2 and 3 days. Finasteride and dutasteride were used as positive control. Cell Counting Kit-8 (CCK8) was used to evaluate the cell proliferation. In general, 10μl CCK8 solution was added to each plate and cells were incubated for 2 h at 37°C. The cell viability was revealed by the absorbance which was measured at 450 nm. All experiments were performed three times and the mean absorbance values were calculated. The results are expressed as a percentage of absorbance caused by treatment compared to those of the vehicle treated controls.

mRNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR)

RNeasy Mini Kit (Qiagen, Germany) was used to extract mRNA from cells in accordance with the manufacturer's instructions. Then NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) was applied to determine the concentration of mRNA. Reverse transcription of total mRNA was performed at 1 μg of total mRNA in 25 μl final volume using random primers. After reverse transcription, the cDNA concentration was also determined.

Quantitative real-time PCR was performed using Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument (Thermo Scientific, USA). Primers (GENEWIZ, SuZhou, China) used for PCR reactions are listed in Table 2. Primer sequences were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Each reaction mix was prepared using 10 μl 2× Real time PCR Master Mix (SYBR Green), 7ul DEPC-treated Water, 4 μl of cDNA template with 1 μM of each primer in a total reaction volume of 20 μl. The PCR was run for 40 cycles and the thermal cycling conditions were as follows: initial heat activation at 95ºC for 10 min; denaturation for 10 s at 95ºC; and combined primer annealing and extension for 60 s at 60ºC. The fluorescence signal was measured at the end of each extension step. Fluorescence emission readings were analysed using 7500 software (Thermo Scientific, USA). The data were presented as the relative gene expression (%) of the target gene expression, normalized to the housekeeping gene GAPDH, compared to the non-treated group.

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
SRD5A1	CTACGGGCATCGGTGCTTAATTTAC	AAGCCAAAACCTATTAGAAAACGGG
SRD5A2	CTACGGGAAGCACACGGAGAG	CACAAATGTCCTGTGGAAGTAATGT
AR	ACATGCGTTTGGAGACTGCC	TACCAAGTTTCTTCAGCTTCCGGG
ER	TGGGAATGATGAAAGGTGGGAT	GGTTGGCAGCTCTCATGTCT
DKK-1	TGACAACTACCAGCCGTACC	CAGGCGAGACAGATTTGCAC
TGF-β1	CCTTCCAAGGCAAGTTCATGG	TGGCAAATTACCACTCGGAAGT
TGF-β2	CTACCTGCAGCACACTCGAT	CAACTGGGCAGACAGTTTCG
IGF-1	GGATGAGCTTCTACAACGCGA	TTCTGGATGCCTTTGCCCAT
GAPDH	AAATCCCATCACCATCTTCC	ATGACCCTTTTGGCTCCC

Table. 1: DNA sequence of primer pairs used for quantitative real time PCR.

Statistical Analysis

All results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Student's t-test was used to determine the statistical significance (p-value <0.05). SPSS software was used for statistical analysis.

Results

The Effects of OA on 5α-Reductase Activities

We performed an in vitro cell based 5α-reductase assay based on a previously established method[15]. Prior to the analysis of the samples, a standard calibration plot was done with five concentrations of DHT. DHT showed good linearity over the concentration range of 0-40 ng/ml with a correlation coefficient of $r^2 \geq 0.99$. The typical regression equation of DHT was $y = 0.1173x + 0.2106$.

We investigated the effects of OA on the 5α-reductase activity using rat prostatic and hepatic crude enzymes, which mainly contain type II 5α-reductase and type I & II 5α-reductase, respectively. As shown in Fig.1, OA significantly inhibited 5α-reductase activities in a dose-dependent manner, markedly inhibited prostatic 5α-reductase activities by 22.3-90.9% and hepatic 5α-reductase activities by 17.4-90.1%. Especially, when the reaction mixture was incubated with 8-16 mg/ml of OA, its inhibition activity was similar to that of the finasteride and dutasteride treated group, two positive control.

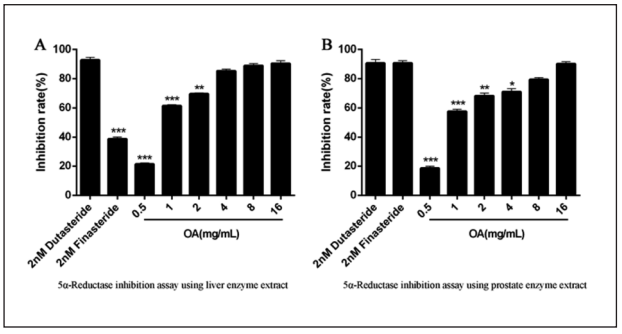

Figure 1: Inhibition effects of on 5α-reductase activities.

Finasteride, a positive control, is a specialized type II 5α-reductase inhibitor, inhibited prostatic 5α-reductase activity by above 92.4% at a 2 nM concentration; however, there was a weak inhibitory effect on hepatic 5α-reductase activity. OA could inhibit both type I and II 5α-reductase activity effectively, which suggests that OA has an advantage over finasteride.

Assay of 5α-reductase inhibition was performed using a crude enzyme extract of rat prostate and liver. The reaction mixture contained testosterone, prostatic or hepatic crude enzyme and OA. The conversion rate of testosterone (T) to dihydrotestosterone (DHT) was calculated by the equation [DHT/(T + DHT)]. Inhibition activity (%) was expressed as a percentage of reduced conversion rate compared to the control. The inhibition activity of control group was regarded as 0% (not shown). Dutasteride was used as a positive control. Data are presented as the mean ± SD of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. positive control.

The Effects of OA on the Proliferation of Dermal Papilla Cells

We examined the effects of OA on the proliferation of DPCs. When DPCs were treated with OA at concentrations of 0.0001, 0.001, 0.01, 0.1 and 1μM, OA increased the proliferation of DPCs by 113.1%, 122.4%, 141.9%, 126.8% and 105.7%, respectively, and significantly promoted the proliferation of DPCs compared with the control at 0.001, 0.01 and 0.1μM concentrations at 72h (Fig. 2). These results suggest that OA might promote hair growth effects by increasing the proliferation of DPCs.

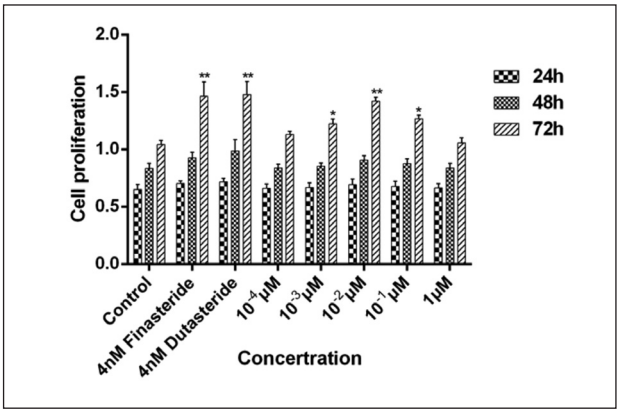

Figure 2: The effects of oleanolic acid on the Proliferation of DPCs.

DPCs were plated in 96 well plates and treated with various concentrations of OA for 4 d. Cell proliferation was measured by the CCK8 assay. All experiments were performed in triplicate. Finasteride and dutasteride (4nM) was used as positive control. Data are presented as the mean±S.D. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs Control.

Quantitative Real-Time PCR Analysis

Real-time polymerase chain reaction (RT-PCR) was performed to assay the expression of nine genes of DPCs. We measured the expression levels of SRD5A1, SRD5A2, AR, ER, DKK-1, IGF-1, TGF-β1,

TGF-β2, VEGF and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The gene expression levels were normalised to the housekeeping gene (GAPDH) and expressed as a percentage of the control group. For selected genes that were involved in the androgen signalling pathway, SRD5A1 and SRD5A2 was significantly downregulated ($p<0.05$) after OA treatment by 49% and 18% respectively, the expression of AR was significantly reduced ($p < 0.05$) by 49%, while the expression of ER was significantly increased ($p < 0.01$) by 72% (Fig. 3A-D). Selected key growth factor genes that play essential roles in hair cycle regulation, primarily IGF-1, TGF-β1 and TGF-β2 showed significant changes in expression levels after treatment with OA. IGF-1 showed a 164% increase ($p< 0.01$) in expression levels after treatment with OA (Fig. 3G), while TGF-β1 and TGF-β2 showed 66% and 27% decreases ($p< 0.05$) in expression levels after being treated with OA (Fig. 3E-F), respectively. VEGF showed a 124% increase ($p<0.01$) in expression levels after treatment with OA (Fig. 3H). DKK-1 was downregulated by 14% (Fig. 3I); however, no statistical significance was found.

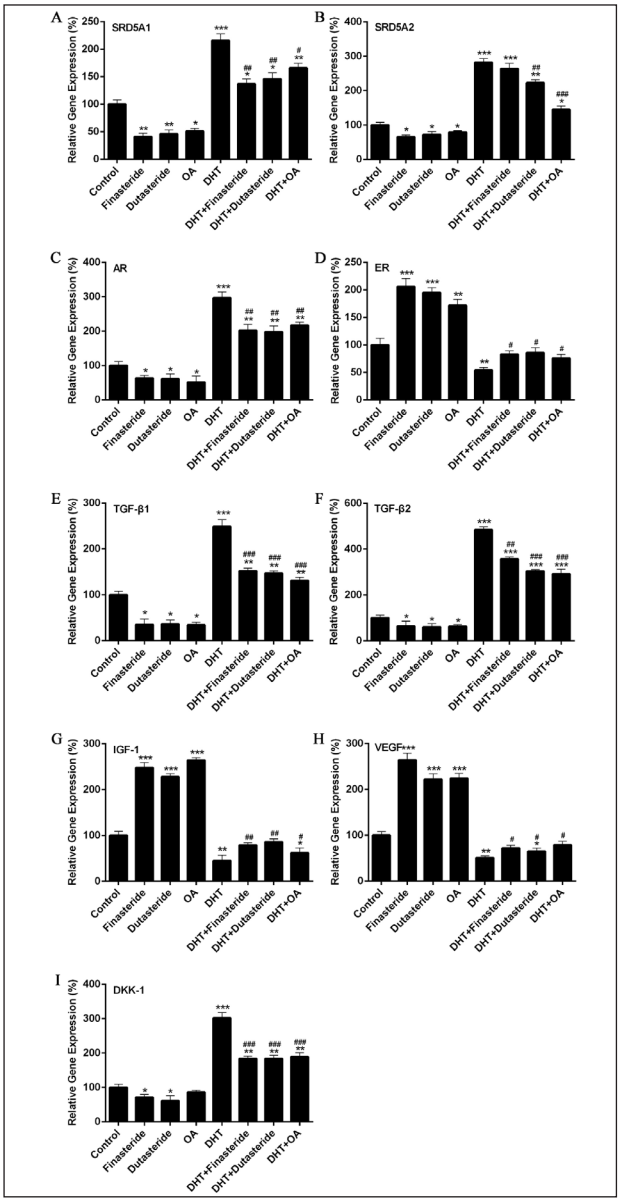

Figure 3: The effect of OA on (A) 5α-reductase I (B) 5α-reductase II (C) androgen receptor (D) oestrogen receptor (E) TGF-β1 (F) TGF-β2 (G) IGF-1 (H)VEGF and (I) DKK-1 gene expression of DPCs after 48h treatment. .

The gene expression was analysed using real-time PCR. The level of gene expression in the non-treated (control) group was set to 100%. The graphs summarise the experimental analyses (mean ± SD). Data are presented as the mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. DHT treated group.

DHT had a significant influence on the gene expression of DPCs, up-regulated SRD5A1, SRD5A2, AR, DKK-1, TGF-β1, and TGF-β2 expression, and down-regulated ER, IGF-1, VEGF expression. OA effectively inhibited the effect of DHT on DPCs gene expression similarly to finasteride and dutasteride (Fig. 3A-I).

Discussion

Previous studies about OA are mainly concerning its bioactive anti-inflammatory, anti-oxidative, antiprotozoal, anti-mutagenic or anticancer properties; much less attention has been paid to its anti-androgenic effects. Our previous research found that OA, identified in 55 Chinese herbal medicines, could promote hair growth in vitro, specifically accelerating the growth of hair shafts and prolonging the growth period. However, the underlying mechanisms of OA promoting hair growth have not been described until now. To further understand this herbal ingredient, this study has investigated the anti-androgenic effects of OA.

DHT can cause AGA in several different ways, first DHT induce the miniaturization of dermal papilla and hair follicles, which leads to transition from anagen to catagen[16]; second DHT increases the levels of TGF-β1 and TGF-β2 in DPCs, which leads to decreased proliferation of epithelial cells[17-18]; third DHT up-regulate of Dickkopf related protein-1 (DKK-1) could cause repression of the growth of epithelial cells in hair follicles[19]. Therefore, the inhibition of 5α-reductase has the effect of improving hair loss in AGA patients. Now, finasteride is used as mainstream therapeutic to inhibit the enzyme 5α-reductase and reach lower plasma

levels of DHT circulating in the bloodstream[20]. In our study, OA was shown to possess activity against 5α-reductase in vitro reaction and reduce the production of DHT. OA significantly inhibited 5α-reductase activity in a dose dependent manner, when the concentration increased to 8-16 mg/ml, its inhibition activity of type II 5α-reductase was similar to finasteride and dutasteride. OA could inhibit both type I and II 5α-reductase activity effectively, which suggest that OA have an advantage over finasteride.

The depletion of DPC number was believed to force the hair follicle to experience a premature anagen phase (growth phase), shorten it from an average of 2-7 years to just several months. So regulation of hair growth depends on the balance between proliferation and apoptosis in the DPC[21]. Recently, many studies have used DPC proliferation as a preliminary assessment for DPC growth inducers and a model for evaluating their potential as anti-AGA agents[22]. In our study, DPC proliferation was measured using the CCK8 assay to determine the number of viable cells remaining in the culture at three time points with the OA at concentrations 0.0001, 0.001, 0.01, 0.1 and 1 μM. As shown in Fig. 2, OA was shown to effectively induce DPC proliferation at concentrations as low as 0.001μM, and the best concentration is 0.01μM.

DPCs are very important in the regulation of the hair cycle for producing a lot of diffusible proteins, such as IGF-1, VEGF and TGF-β. However, the gene expression of DPCs is influenced by DHT for the levels of TGF-β1, TGF-β2 and DKK-1 increased after DHT treatment. To identify the effect of OA on gene expression of DPCs and determine its anti-DHT effect, nine genes were studied using RT-PCR after DPCs incubation with the OA for 48 h at 0.01μM. Among the selected genes, SRD5A1, SRD5A2 and AR were involved in the androgen signalling pathway, AR and ER were most intensely expressed in human scalp DPCs[23], IGF-1, TGF-β1 and TGF-β2 were important genes regulating human hair cycle (24-26), DKK-1 as one of the most upregulated DHT-inducible genes in DPCs[19] involved in hair induction and growth,[27] VEGF was known to increase the cell proliferation of DPCs.

In our findings, we observed that OA significantly upregulated the production of IGF-1, ER, and VEGF by 164%, 72%, 124%, respectively, and downregulated the production of SRD5A1, SRD5A2, AR, TGF-β1, TGF-β2 and DKK-1 by 49%, 18%, 49%, 66%, 27% and 14%, respectively, in DPCs. Moreover, OA effectively inhibited the effect of DHT on DPCs gene expression similarly to finasteride and dutasteride. Hence, OA may potentially be able to act as an agent against DHT. The dramatic increase of IGF-1 by OA may be a promising sign that OA could potentially be used as an agent to restore the IGF-1 levels in AGA patients.

Conclusion

OA exhibited good inhibitory activity against 5α-reductase in vitro, could promote DPC proliferation, reduced the expression of six upregulated genes in AGA, increase three downregulated genes in AGA, and reduced the impact of DHT on DPC gene expression. The results suggest that OA could have potential as a treatment for AGA via the inhibition of 5α-reductase activities, thereby promoting the proliferation of DPCs, and altering the gene expression of DPCs.

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Corresponding Author:
WeiXin Fan
Email: 13327805737@189.cn
(China)