



The extract of *Thujae occidentalis* semen inhibited 5α -reductase and androchronogenetic alopecia of B6CBAF1/j hybrid mouse

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KEYWORDS

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Summary Background: The conversion of testosterone to dihydrotestosterone; 5α -androstan- 17β -ol-3-one by 5α -reductase plays a crucial role in hair baldness and prostatomegaly. Recent approach showed specific inhibitors for 5α -reductase type 2 such as finasteride promoted hair growth in male pattern alopecia. **Objective:** In order to search for effective medicinal plant extracts applied topically for androgenetic alopecia, we screened natural plant extracts having inhibitory activities of 5α -reductase type 2 and demonstrated its biological function in androgen-related animal models. **Methods:** We evaluated the inhibition activities of numerous plant extracts by contact cell based metabolic method using a stable HEK 293 cell line expressing human 5α -reductase (type 2). To elucidate the biological activity in vivo, the *Thujae occidentalis* semen (TOS) extract was topically applied to fuzzy rat and androchronogenetic alopecia (AGA) mouse, respectively. The secreted sebum and the size of sebaceous glands of fuzzy rat were measured after 6 weeks. Also, after the topical treatment with TOS extract and androgen receptor antagonist (cyproterone acetate) simultaneously with subcutaneous injection of testosterone (1 mg/mice/day), hair loss patterns of female B6CBAF1/j hybrid mouse were observed. **Results:** TOS extract showed higher inhibition activity of 5α -reductase type 2 (IC_{50} value = 2.6 μ g/ml) than that of γ -linolenic acid, but lower than that of finasteride. When applied to fuzzy rat, the amount of sebum and sebaceous gland size decreased remarkably. In AGA model, alopecia degrees of two groups, treated with TOS extract ($P < 0.015$) or cyproterone acetate ($P < 0.01$), were lower than that of vehicle (propylene glycol:ethanol = 7:3) and there was no difference between above two groups. **Conclusion:** We have demonstrated the inhibitory activity of TOS extract for 5α -reductase type 2 and its biological action in two animal models, suggesting that TOS extract would be used as an effective agent for male pattern baldness by modifying androgen conversion.

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1. Introduction

Δ^4 -3-oxo-steroid 5α -oxidoreductase (EC 1.3.99.5; 5α -reductase) is a microsomal enzyme

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that converts testosterone into the more potent intracellular androgen 5α -dihydrotestosterone (DHT) [1,2], which then binds to androgen receptor to exert its biological function [3]. Because DHT has a high binding affinity to androgen receptor among many androgens, it plays an important role in the generation of male pattern alopecia [4], prostate cancer [5], benign prostatic hyperplasia [6], acne [7], and female hirsutism [8]. For the treatment of androgen-related disorder, synthetic 5α -reductase inhibitors such as steroidal finasteride were developed, and some natural nonsteroidal compounds have been reported, for example, (–)-epigallocatechin-3-gallate [9], chlorophorin [10], bisnaphthoquinone derivative [11] and γ -linolenic acid [12].

As human tissue e.g. follicle and prostate is limited in availability, recombinant human embryonic kidney 293 cell line expressing stable 5α -reductase was established by transfecting cDNAs for the two isoforms [13]. In situ metabolic inhibition assay was performed using the transfected cell lines to test natural plant extracts as well as the finasteride.

In our search for 5α -reductase inhibitor from oriental medicinal plants, we found that the extract of *Thujae occidentalis* semen (TOS) inhibited 5α -reductase in vitro. The leaves and the fruits of *Thujae occidentalis* have been known as well to serve as an oriental herbal medicine for the treatment of renopathy, leukotrichia and alopecia [14].

The extract of TOS also applied to two rodent models that were reported to show physiological appearances susceptible to androgens. The first fuzzy rat model, a genetic mutant between hairless and hairy albino rat, expresses androgen-dependent hypersecretion of sebum and hyperplastic sebaceous glands [15]. The second testosterone inducible model of alopecia, called the androchronogenetic alopecia (AGA), has been reported in B6CBAF1/j mice that were a hybrid cross between a C57BL/6 female and a CBA male [16,17]. The in vivo activity of the extract for 5α -reductase was elucidated in both the fuzzy rat model related to sebaceous gland and the AGA mouse model.

2. Material and method

2.1. Preparation of TOS extract

One kilo gram of TOS were extracted with 2 l of 95% ethanol using distiller with a reflux condenser for 4 h and concentrated using vacuum evaporator. Nonpolar fraction of this extract was obtained by

repeated extraction with ethyl acetate (1 l), and the sample was concentrated in vacuo, giving residue (designated TOS extract) of 5.2 g.

2.2. Construction of stable HEK293 clone expressing 5α -reductase activity

Two pCMV7- 5α R plasmids, which was kindly provided by Russel, contain full length human cDNAs encoding both 5α -reductase isozymes [18]. pCMV7- 5α R2 plasmid was used in the PCR reaction with the primers designed to introduce *EcoRI* site for 5' and *XbaI* site for 3' ends of the 5α -reductase type 2 coding sequence. The primers designed were LCHP-4 (5'-CTA CCA GTA TCG AAT TCG ATG CAG GTT CAG TGC CAG CAG AGC-3') and LCHP-6 (5'-CTA CCA GTA GCT CTA GAT TAA AAG ATG AAT GGA ATA AGG GC-3'). The PCR reaction produced a single band of 0.82 kb, corresponding to the 5α -reductase type 2 gene. The PCR product was then cloned into downstream of the CMV promoter of the eucaryotic expression vector p3 \times FLAG-CMV-10 (Sigma Chemical, USA). The resulting plasmid (p3 \times FLAG-CMV- 5α R2; 7.2 kb) was used for transfection. Transfection to HEK293 cells was performed using lipofectamine plus (Life Technologies, USA) by manufacturer's guidance. Untransfected cells were sacrificed by replacement of medium containing G418 sulfate (400 μ g/ml) and stable single cell clones were picked and identified by following DHT conversion assay and western blotting with anti-FLAG Tag (Sigma Chemical, USA) and anti- 5α -reductase type 2 (Biogenesis Ltd, UK) antibodies.

2.3. In situ metabolic inhibition assay

5α -Reductase activity was determined with intact HEK293- 5α R2 cells by measuring the conversion rate of [14 C]testosterone to [14 C]dihydrotestosterone. HEK293 cells transfected with p3 \times FLAG-CMV- 5α R2 were seeded in a 24 well culture plate at a concentration as 2.5×10^5 cells/well before 1 day. The medium was then removed by aspiration and replaced by 0.5 ml of a freshly prepared substrate/inhibitor medium. This medium consisted of serum-free DMEM medium with 0.05 μ Ci [14 C]testosterone (Amersham Pharmacia biotech, UK). For inhibition studies, an appropriate concentration of samples dissolved in dimethyl sulfoxide was added to the medium solution at a final concentration of 1%. After 2 h incubation at 37 $^{\circ}$ C, 5% CO₂, the mediums were sampled and steroids were extracted with 800 μ l of ethyl acetate. The upper organic layer was separated and evaporated to dryness. The residue was dis-

solved in 50 μ l ethyl acetate and subjected to TLC plate (Silica gel 60F₂₅₄, Merck, Germany). The different molecular species were separated by thin layer chromatography on silica plate in a solvent system consisting of ethyl acetate/hexane (1:1) and the separate spots were quantified by phosphoimaging (FLA-3000, Fuji Film, Japan) using the MacBas software package. The conversion activity of 5 α -reductase was expressed as the ratio DHT/(T + DHT) [19]. The IC₅₀ values of the samples were obtained from at least duplicate determinations.

2.4. Androgen-related hypersecretion of sebum in male fuzzy rat

The 1% TOS extract solution was prepared in the vehicle (EtOH:Propylene glycol:Water = 5:3:2). Six male fuzzy rats (Charles River Laboratories, USA) were divided into three groups, which consisted of untreated group (a), vehicle-treated group (b) and 1% TOS extract-treated group (c). Approximately 0.5 ml of the above solution or vehicle was topically applied to back skins twice per day 5 days per week in animals of the respective groups. After 6 weeks of the beginning treatment, the animals were anesthetized with pentobarbital at the dose of 40 mg/kg intraperitoneally and photographs of the backs of the rats were taken and the fresh skin tissues were fixed with 10% neutral buffered formalin. Skin samples were taken by punch (8-mm diameter) and stained with hematoxylin and eosin to observe morphology of sebaceous glands.

2.5. Androgen mediated alopecia model with B6CBAF1/j hybrid mouse

B6CBAF1/j hybrid mouse (AGA mouse) was purchased from Jackson Laboratory (USA) and maintained and bred under conventional conditions. Female AGA mice were injected daily with testosterone (Fluka Chemical, Germany) (suspended in an aqueous carboxymethylcellulose vehicle) at a dose of 1 mg/day. Injections were given once daily 5 days a week for 12 weeks. Simultaneously, 200 μ l of the 1% TOS extract solution or the other control solutions was applied on the upper dorsum once a day for 12 weeks. Control groups consisted of the group of mice injected with testosterone only (a), the group of mice injected with testosterone and given topical application of vehicle (b) and the group of mice injected with testosterone and given topical application of 50 μ l of cyproterone acetate (Sigma Chemical, USA) (c). At the end of the study,

the animals were sacrificed by cervical dislocation. The pattern of hair loss was evaluated using a grading system of 0–4 as previously described [20]. Upper and lower dorsum samples of mouse were taken and stained as described in fuzzy rat method.

3. Results and discussion

3.1. Stable HEK293 cell lines expressing 5 α -reductase type 2

The selected G418 sulfate-resistant HEK293 single cell clone was identified by western blot and immunocytochemistry analysis using two antibodies for anti-FLAG Tag and anti-5 α -reductase type 2 after metabolic enzyme assay. In western blot analysis, single band was observed at 30 kDa using above two antibodies (Data not shown). Targeted 5 α -reductase isozyme was detected in nucleus of HEK293 cells in immunocytochemistry using same antibodies for western blot analysis.

K_M value of expressed 5 α -reductase was determined in whole cell based metabolic assay by Lineweaver-Burk plot analysis. [¹⁴C] Testosterone has been used as a substrate of the range from 0.35 to 3500 μ M. The kinetic K_M value for testosterone affinity was 0.93 μ M which was higher than reported value (0.56 μ M; Reichert et al. [21]).

Above HEK293 cell line expressing stably the 5 α -reductase type 2 could be easily used to test inhibitors of the enzyme, since it can be kept frozen and be re-cultivated.

3.2. In situ metabolic inhibition test using stable HEK293-5 α R2 cell line

Constructed HEK293-5 α R2 cells were used to estimate inhibition activities of plant extracts, the reported steroidal and non-steroidal inhibitors. Whole cells were incubated with 0.05 μ Ci of radiolabeled testosterone and the test sample at 37 °C and 5% CO₂ gas for 2 h. We compared the DHT conversion activities of HEK 293-5 α R2 clones (transfected) with HEK293 cell lines (untransfected) (Fig. 1). The DHT conversion ratio (DHT/(DHT + Testosterone)) of HEK 293-5 α R2 clones was linear until 6-h incubation and maximum conversion ratio was measured about 90%, but we could not observe the DHT conversion activity of untransfected HEK 293 cells (Fig. 1).

Among the herbal medicinal plants tested, TOS extract showed a highest inhibition activity. The TOS was known to contain much essential oils, so we decided to compare its inhibition activity with

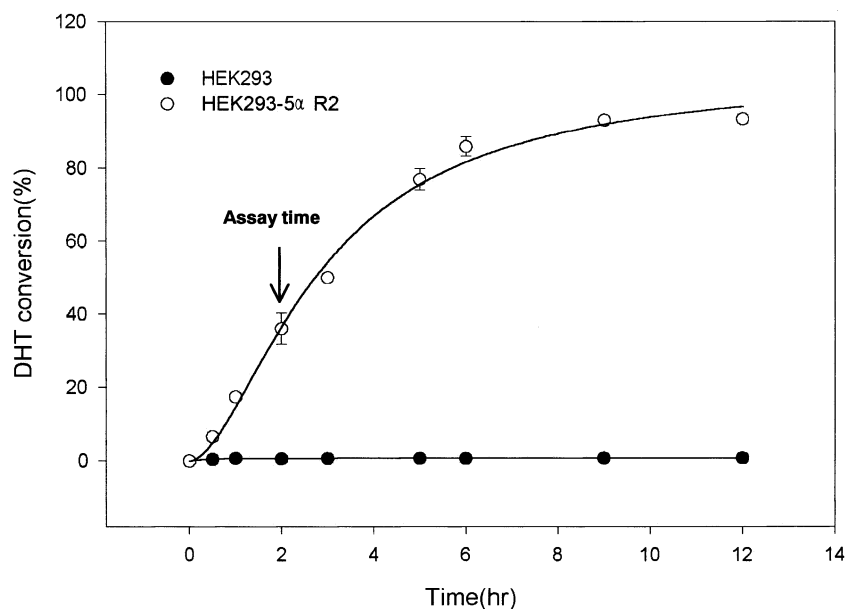


Fig. 1 Two 5 α -Reductase (type 2) activities of two untransfected HEK293 cells and HEK293 cells transfected with p3 \times FLAG-CMV-5 α R2. The enzyme activity was exhibited as DHT conversion ratio (T/(T + DHT)*100). In situ metabolic incubation time for assay was determined at 2 h, which showed a linear conversion rate (Mean \pm SD).

Table 1 IC₅₀ values of selected compounds and the extract, which were determined in tact HEK293-5 α R2 cell line

Compound	IC ₅₀ values	Reference
Linoleic acid	31.55 μ g/ml (112.50 μ M)	
γ -Linolenic acid	5.55 μ g/ml (19.93 μ M)	3.3 μ M ^a
TOS extract	2.6 μ g/ml	
Finasteride	0.34 μ M	0.57 μ M ^b

^a According to reference of Liang et al. [12].

^b According to reference of Reichert et al. [13].

those of unsaturated fatty acids in addition to potent steroidal inhibitor, finasteride. The IC₅₀ values were determined as described above (Table 1).

As expected, finasteride showed most potent inhibition activity, which showed a little lower IC₅₀ value than that of reported previously [21]. Unsaturated fatty acid such as linoleic acid, γ -linolenic acid, showed lower inhibition activities than that of TOS extract. Highly saturated fatty acid had no inhibition (Data not shown), which indicated the saturation degree of fatty acid is in reciprocal proportion with enzyme inhibition. As a selected ethyl acetate fraction of TOS extract showed lower IC₅₀ value than those of fatty acids, we supposed that there are likely to be unknown active compounds in addition to oil component.

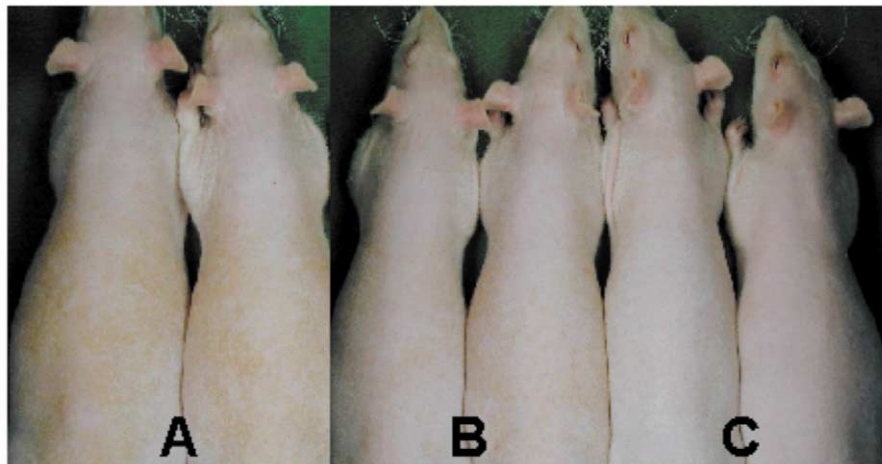
3.3. Effect of TOS extract on sebaceous glands in male fuzzy rat

Using male fuzzy rat model exhibiting androgen-dependent hyperplasia of the sebaceous glands, we

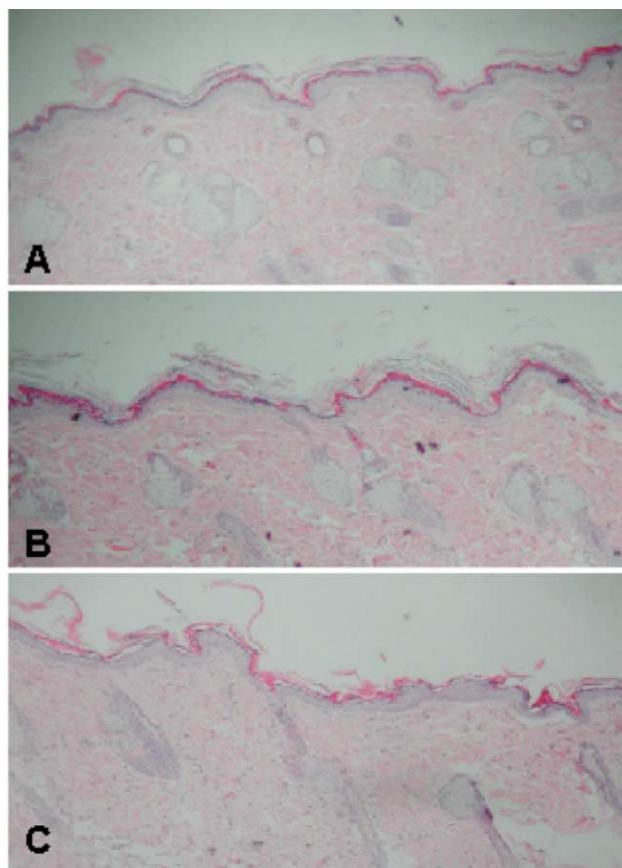
Table 2 Effect of the topical application of cyproterone acetate (CA) and TOS extract on hair loss of the AGA mouse

	Index of alopecia		
	Week 0	Week 10	Week 12
Testosterone only	0	1.80 \pm 0.57	2.12 \pm 0.52
Testosterone + vehicle	0	0.70 \pm 0.27	1.04 \pm 0.09
Testosterone + CA	0	0.26 \pm 0.23*	0.22 \pm 0.18*
Testosterone + TOS Ext.	0	0.22 \pm 0.04**	0.54 \pm 0.29

Mean \pm SD of seven animals per groups. * P < 0.01, ** P < 0.015 compared with vehicle-treated group with paired t -test.

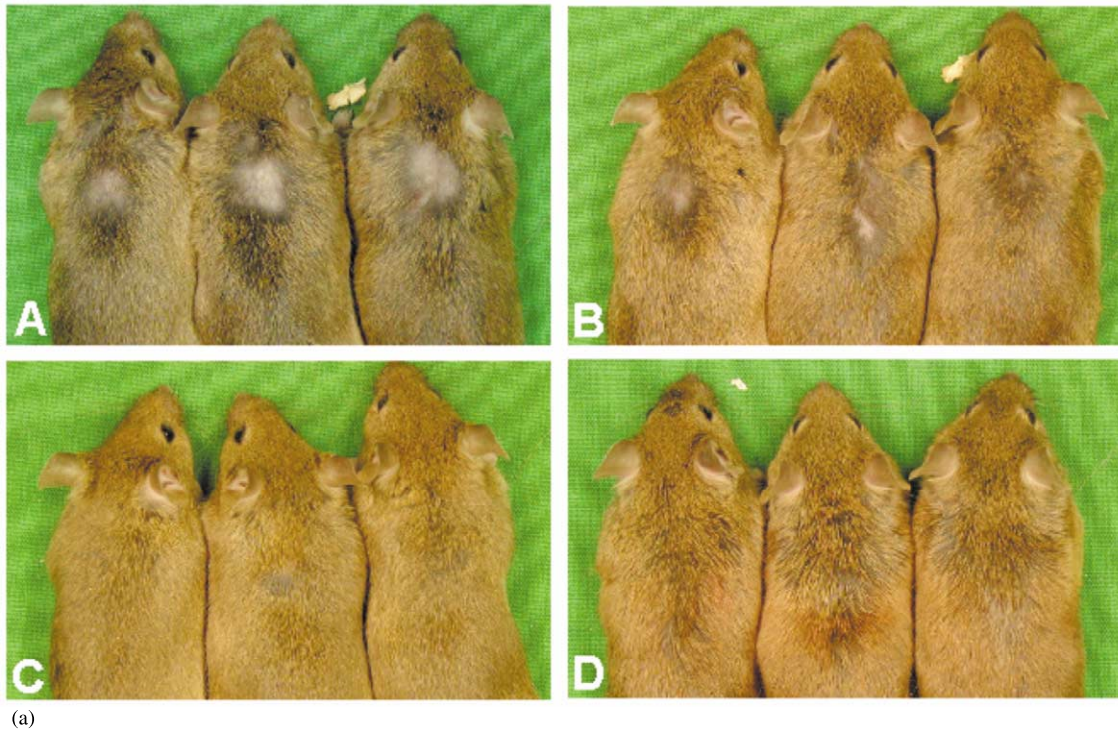


(a)

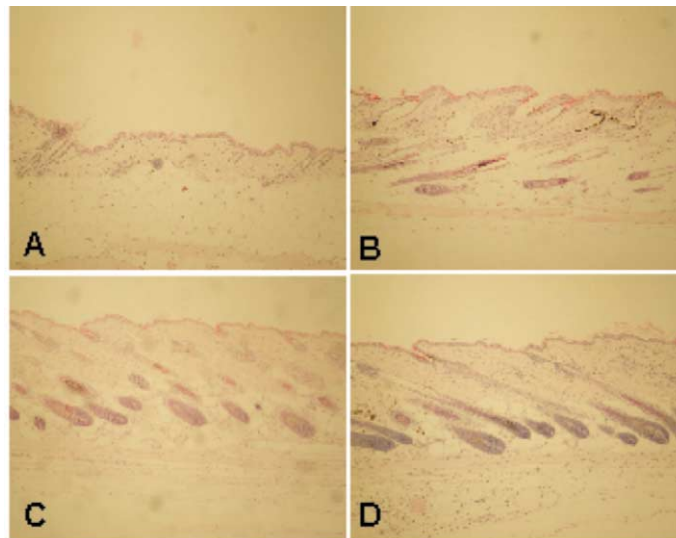


(b)

Fig. 2 Inhibition activity of the TOS extract on seborrhea and the size of sebaceous glands of fuzzy rat. Lower secretion content of dark brownish-colored seborrhea and smaller sebaceous gland were observed in the group applied topically 1% TOS extract (C) in comparison with two groups of untreated (A) and treated with vehicle (B, ethanol:propylene glycol:water = 5:3:2). Histological observation in cross tissue section of each group stained with hematoxylin and eosin (60 \times magnitude).



(a)



(b)

Fig. 3 Comparison of baldness patterns in the AGA mouse after 12 weeks. A is the group injected only testosterone subcutaneously (1 mg/mouse/day), B, C and D are the groups applied topically vehicle (propylene glycol:ethanol = 7:3), 5% cyproterone acetate (CA) and 1% TOS extract after testosterone subcutaneous injection, respectively. (a) Photographs of the AGA mice of each group, (b) histological observation in cross tissue section of each group stained with hematoxylin and eosin (50 × magnitude).

observed the topical effects of TOS extract. Finasteride, known as a specific inhibitor for 5 α -reductase type 2, significantly suppressed the size of the sebaceous glands in previous study [15]. At the time of beginning of treatment (3 weeks of age), short white hair covered the entire body surface. At the time of experiment termination (6th week of treatment), the backs of the untreated group exhibited the most pronounced seborrhea (Fig. 2a). The back skin of the TOS extract-treated group showed less seborrheic appearance compared to the vehicle-treated and untreated groups. But there was no difference of body surface hairs (more sparse hair than initial state). We could not observe the correlation of hair growth and sebaceous gland size in three groups.

In histological observation, the sizes of glandular lobe and sebaceous duct of the group treated with 1% TOS extract decreased in comparison to those size in untreated and vehicle-treated animals although the differences of each size was not measured with image analysis program (Fig. 2b).

When TOS extract was applied systemically to fuzzy rat (300 mg/kg), we could not find the same effect suppressing seborrhea and there was no change of body weight and prostate size in both animal groups when compared with vehicle (saline) treated group.

Anti-androgen effect of the TOS extract was examined in hyperactive sebaceous glands of fuzzy rat, which indicated that the extract would be used as an agent for skin disorder such as acne and androgenetic alopecia.

3.4. Effect of TOS extract on alopecia of female B6CBAF1/j hybrid mouse (AGA mouse)

To apply the TOS extract to specific androgen-related baldness process, we treated the plant extract to the female AGA mouse for 12 weeks with concomitant injection of testosterone at dose of 1 mg/day as previously described. In AGA mouse, potent dihydrotestosterone was reported to induce an earlier onset of hair loss [20], so we supposed that 5 α -reductase inhibitor would delay the hair loss induced by testosterone injection. Androgen-dependent diffuse baldness of AGA mouse was induced at upper dorsal skin within 6 weeks of testosterone treatment, which time was later than the time reported by Matias et al. [16].

Hair loss of AGA mouse was determined on 10 and 12 weeks after beginning treatment (Table 2). Statistically significant differences with vehicle-treated group were observed at both the groups

treated with 5% cyproterone acetate (C) and 1% TOS extract (D) at two determined evaluations times.

The alopecia pattern was not found at the groups treated with 1% TOS extract (D) or 5% cyproterone acetate (C), although the animal group treated only testosterone injection (A) and the group treated vehicle after testosterone injection (B) showed a dispersed hair loss at dorsal area (Fig. 3a). No anagen hair follicles were seen in the A and B group, whereas anagen follicles were observed in upper dorsum section of mouse in group C and D (Fig. 3b). Also the sebaceous glands of the skin sites that normally become bald (upper dorsum) were larger in comparison with the nonbalding site (lower dorsum) (data not shown).

Because testosterone causes a prolonged telogen in the AGA mouse, it is likely that this rodent model could be used a effective therapeutic approach for the test of anti-androgen drugs such as receptor antagonist and 5 α -reductase inhibitor besides vasoactive agent (minoxidil).

We elucidated the 5 α -reductase inhibition activity of TOS extract both in whole cell-based level and in vivo animal studies, so the extract could be used for the treatment of skin disorder related to androgen action such as male pattern alopecia, female hirsutism [8,22].

In this study, we showed that TOS extract has a inhibitory effect for only type 2 5 α -reductase. But there are two isoforms of 5 α -reductase in hair follicle which are different enzyme kinetic parameters and chromosomal localization [23], so it is necessary to observe the inhibition effect for type 1 5 α -reductase in future.

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References

- [1] Anderson KM, Liao S. Selective retention of dihydrotestosterone by prostatic nuclei. *Nature* 1968;219:277–9.
- [2] Bruchovsky N, Wilson JD. The conversion of testosterone to 5 α -androstano-17 β -ol-3-one by rat prostate in vivo and in vitro. *J Biol Chem* 1968;243:2012–21.
- [3] Liao S, Kokontis J, Hiipakka RA. Androgen receptors: structures, mutations, antibodies and cellular dynamics. *J Steroid Biochem* 1989;34:41–51.
- [4] McPhaul MJ, Young M. Complexities of androgen action. *J Am Acad Dermatol* 2001;45(Suppl 3):S87–94.
- [5] Bruckheimer EM, Kyprianou N. Dihydrotestosterone enhances transforming growth factor-beta-induced apoptosis

- in hormone-sensitive prostate cancer cells. *Endocrinology* 2001;142:2419–26.
- [6] Bartsch G, Rittmaster RS, Klocker H. Dihydrotestosterone and the concept of 5-alpha-reductase inhibition in human benign prostatic hyperplasia. *Eur Urol* 2000;37:367–80.
- [7] Forstrom L. The influence of sex hormones on acne. *Acta Derm Venereol* 1980;89(Suppl):27–31.
- [8] Faloi E, Filipponi S, Mancini V, Di Marco S, Mantero F. Effect of finasteride in idiopathic hirsutism. *J Endocrinol Invest* 1998;21:694–8.
- [9] Liao S, Hiipakka RA. Selective inhibition of steroid 5-alpha-reductase isozymes by tea epicatechin-3-gallate and epigallocatechin-3-gallate. *Biochem Biophys Res Commun* 1995;214:833–8.
- [10] Shimizu K, Fukuda M, Kondo R, Sakai K. The 5-alpha-reductase inhibitory components from heartwood of *Artocarpus incisus*: structure–activity investigations. *Planta Medica* 2000;66:16–9.
- [11] Ishiguro K, Oku H, Kato T. Testosterone 5-alpha-reductase inhibitor bisnaphthoquinone derivative from *Impatiens balsamina*. *Phytother Res* 2000;14:544–6.
- [12] Liang T, Liao S. Inhibition of steroid 5-alpha-reductase by specific aliphatic unsaturated fatty acids. *Biochem J* 1992;285:557–62.
- [13] Reichert W, Hartmann RW, Jose J. Stable expression of the human 5-alpha-reductase isoenzymes type I and type II in HEK293 cells to identify dual and selective inhibitors. *J Enzyme Inhib* 2001;16:47–53.
- [14] Kim DK. *The textbook of pharmacognosy*. Seoul: Dongmyong, 2002.
- [15] Ye F, Imamura K, Imanishi N, Rhodes L, Uno H. Effects of topical antiandrogen and 5-alpha-reductase inhibitors on sebaceous glands in male fuzzy rats. *Skin Pharmacol* 1997;10:288–97.
- [16] Matias JR, Malloy V, Orentreich N. Animal models of androgen-dependent disorders of the pilosebaceous apparatus. 1. The androchronogenetic alopecia (AGA) mouse as a model for male-pattern baldness. *Arch Dermatol Res* 1989;281:247–53.
- [17] Sundberg JP, King LE, Bascom C. Animal models for male pattern (androgenetic) alopecia. *Eur J Dermatol* 2001;11:321–5.
- [18] Andersson S, Russell DW. Structural and biochemical properties of cloned and expressed human and rat steroid 5-alpha-reductases. *Proc Natl Acad Sci USA* 1990;87:3640–4.
- [19] Gerst C, Dalko M, et al. Type-1 steroid 5 α -reductase is functionally active in the hair follicle as evidenced by new selective inhibitors of either type-1 or type-2 human steroid 5 α -reductase. *Exp Dermatol* 2002;11:52–8.
- [20] Matias JR, Orentreich N. The effect of testosterone, cyproterone acetate, and minoxidil on hair loss in the androchronogenetic alopecia mouse. *Clin Dermatol* 1988;6:169–76.
- [21] Reichert W, Michel A, Hartmann RW, Jose J. Stable expression of human 5alpha-reductase type II in COS1 cells due to chromosomal gene integration: a novel tool for inhibitor identification. *J Steroid Biochem Mol Biol* 2001;78:275–84.
- [22] Bayram F, Muderris II, Guven M, Kelestimir F. Comparison of high-dose finasteride (5 mg/day) versus low-dose finasteride (2.5 mg/day) in the treatment of hirsutism. *Eur J Endocrinol* 2001;147(4):467–71.
- [23] Jenkins EP, Hsich C-L, Milatovitch A. Characterization and chromosomal mapping of a human steroid 5 α -reductase gene and pseudogene and mapping of the mouse homologue. *Genomics* 1991;11:1102–12.

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