

Topical Treatment of Hair Loss with Formononetin by Modulating Apoptosis

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Abstract

Formononetin is one of the main components of red clover plants and its role on hair regrowth against hair loss has not been established yet. In the present study, we assessed the potential effects of formononetin on alopecia, along with impaired hair cycles by induction of apoptosis-regression.

Depilated C57BL/6 mice were used for monitoring the hair cycles. Formononetin (1 and 100 μ M) was topically treated to the dorsal skin for 14 days. Topical formononetin treatment induced miniaturized hair follicles to recover to normal sizes. Tapering hair shaft began to grow newly, emerg-

ing from the hair follicles by formononetin. In addition, formononetin inhibited the activation of caspase-8 and decreased the procaspase-9 expression. As a result of formononetin treatment, anti-apoptotic Bcl-2 was up-regulated, whereas pro-apoptotic Bax and p53 were down-regulated, resulting in a decrease of caspase-3 activation. Formononetin showed the obvious inhibition of apoptosis under terminal deoxynucleotidyl transferase dUTP nick end labeling staining thereafter. Taken together, our findings demonstrate that formononetin exerted the hair regrowth effect on hair loss, in which the underlying mechanisms were associated with Fas/Fas L-induced caspase activation, thus inhibiting apoptosis.

Introduction

Hair loss is characterized by abnormal hair cycles. Three phases, anagen (growth), catagen (regression) and telogen (resting) organize the hair cycle and so control the hair development [1]. These dermatologic processes frequently co-occur perifollicular lymphocytic infiltrate-affected anagen, induction of apoptosis-regression catagen and prolonged telogen. Most of all, dystrophic hair follicles affected by the induction of apoptosis in the catagen phase exhibit reduced size and inconsistent pigmentation, resulting in a disabled state of production of hair fiber [2]. These imbalances between the follicle growth and the induction of apoptosis result in hair loss [3].

Some of the hair development promoting drugs against hair loss are capable of transiting from telogen to anagen [4]. Two typical anti-hair loss agents, finasteride and minoxidil, have been reported to induce late anagen from early anagen [5]. Finasteride appears to inhibit type II 5 α -reductase, thereby blocking the conversion of testosterone to dihydrotestosterone and minoxidil is efficacious in transition from the early to late

anagen phase of hair follicles [4,6]. However, finasteride is used for androgenic alopecia male patients and female patients who are past the child-bearing age [7,8]. Minoxidil is known to cause unexpected side effects such as pruritus, dryness, scaling, local irritation and dermatitis [9]. Accordingly, the use of these two famous drugs is limited in spite of its efficacy on promoting hair growth [5]. Alternative treatments are required due to the high relapse rates, varying levels of efficacy and concerns about adverse effects.

Formononetin (7-hydroxy-40-methoxyisoflavone; C₁₆H₁₂O₄) is the main compound of red clover plants *Trifolium pratense* L. (Fabaceae), which belong to herbal isoflavone [10]. The effects of red clover on the scalp hair conditions in post-menopausal women have been reported [11]. However, few reports investigate the effects of formononetin on apoptosis of hair follicle, although numerous studies reported that formononetin has diverse biological effects, including hypolipidemic, anti-oxidant, anti-viral, cardioprotective, immunomodulatory and estrogenic activities [10,12–17]. In addition, a molecular mechanism of formononetin-promoted hair regrowth against alo-

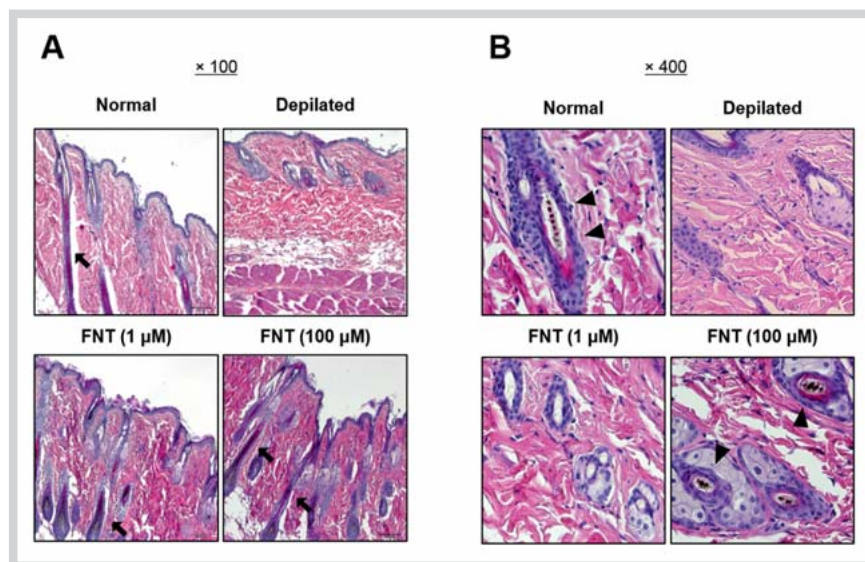


Fig. 1 The effects of formononetin on histological findings by hematoxylin and eosin staining of dorsal skin sections. Arrows and arrowheads indicate to new hair shaft and hair follicles containing hair fibers, respectively. (Color figure available online only.)

pecia has not scientifically proven yet. Therefore, we demonstrated the hair regrowth activities of formononetin against natural hair loss and its mechanism of action in this study.

Results

For 2 weeks, the depilated backs remained hairless in the depilated group. In contrast, mice treated with formononetin showed markedly hair regrowth. By treating formononetin, the area of depilated back skin was covered with black fur. Furthermore, FNT 1 and 100 μM group showed black skin color, while the depilated mice still contained pink or gray skin color. These changes were appeared in histomorphometry. Hair shafts affected by hair loss were broken and fragmented in the depilated group (● Fig. 1A). There were miniaturized hair follicles with no hair fiber (● Fig. 1B). Treatment with formononetin recovered not only the lengths of hair shafts, but also the sizes of hair follicles. Well-straight hair shafts with numerous hair fibers got through the surface of the epidermis after treatment with formononetin. In addition, the size of the dystrophic hair follicles returned to normal levels.

Topical formononetin treatment inhibited the activation of caspase-8 and procaspase-9 (● Fig. 2A). Additionally, formononetin treatment decreased the caspase-3-positive cells and caspase-3 protein levels, while those still were remained in catagen-like depilated hair follicles (● Fig. 3). In mice treated with formononetin, the protein expressions of Bax and p53 were decreased, while the expression of Bcl-2, but not of Bcl- $_{\text{xL}}$, was increased compared to depilated mice (● Fig. 2B,C). These results revealed an increase of Bcl-2/Bax ratio by formononetin.

Numerous cell death pattern in catagen phase of hair cycle were seen in depilated skin region. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells were located in the epithelial cells and surrounded the dermal papilla of hair follicles (● Fig. 4). Formononetin decreased the number of TUNEL-positive cells at the both of treated groups.

Discussion

Well-organized and programmed hair cycles by hair follicles are disrupted by catagen-driven involution, which includes apoptosis [2,18]. Topical formononetin treatment induced hair regrowth in the depilated telogenic C57BL/6 mice, while less visible hair growth was observed in the depilated skin. The lengths of hair shafts and sizes of hair follicles were restored by formononetin. The recovered hair follicles produce visible hair fiber as a result of topical formononetin treatment. In addition, formononetin displayed black skin color, indicating the transition from catagen to anagen, like other anti-hair loss drugs.

Apoptosis requires two crucial events, Fas/Fas ligand (Fas L)-induced caspase activation; extrinsic pathway and mitochondrial cytochrome c (Cyt c) release; intrinsic pathway [19,20]. Fas/Fas L-mediated apoptotic pathway occurs during catagen in the hair follicles, expressing Bcl-2 [1]. The engagement of Fas initiates the cell death pathway [21]. Fas receptor and Fas L recruit Fas-associated death domain (FADD) to form a Fas-FADD complex. These complexes activate caspase-8. Caspase-8 induces caspase-3 activation, while Cyt c from mitochondria is simultaneously released [22]. Its release into the cytoplasm recruits the apoptosis initiator enzyme procaspase-9, thereby facilitating the caspase-3 activation. Thus, extrinsic and intrinsic death pathways are linked to form the active caspase-3, which is essential for apoptosis [23,24]. We observed that formononetin treatment ameliorated apoptosis, indicated by TUNEL-apoptotic cells. Formononetin inhibited the activation of caspase-3 by blocking the caspase-8 and procaspase-9, indicating that formononetin inhibited Fas/Fas L-induced caspase activation and mitochondrial mediated cell death.

In addition to caspase-8 activation, the release of Cyt c into cytoplasm is contributed by Bcl-2 family. It contains two types of proteins, inhibitors of apoptosis (Bcl-2 and Bcl- $_{\text{xL}}$) and promoters of apoptosis (Bax) [25,26]. The balance between anti-apoptotic and pro-apoptotic protein levels is exquisitely orchestrated, and the Bcl-2/Bax ratio is interestingly decreased during catagen stage [27]. Formononetin down-regulated the expressions of Bax, while it up-regulated the expressions of Bcl-2. Therefore, the Bcl-2/Bax ratio was increased by formononetin compared to catagen-like depilated mice. Furthermore, the transcription fac-

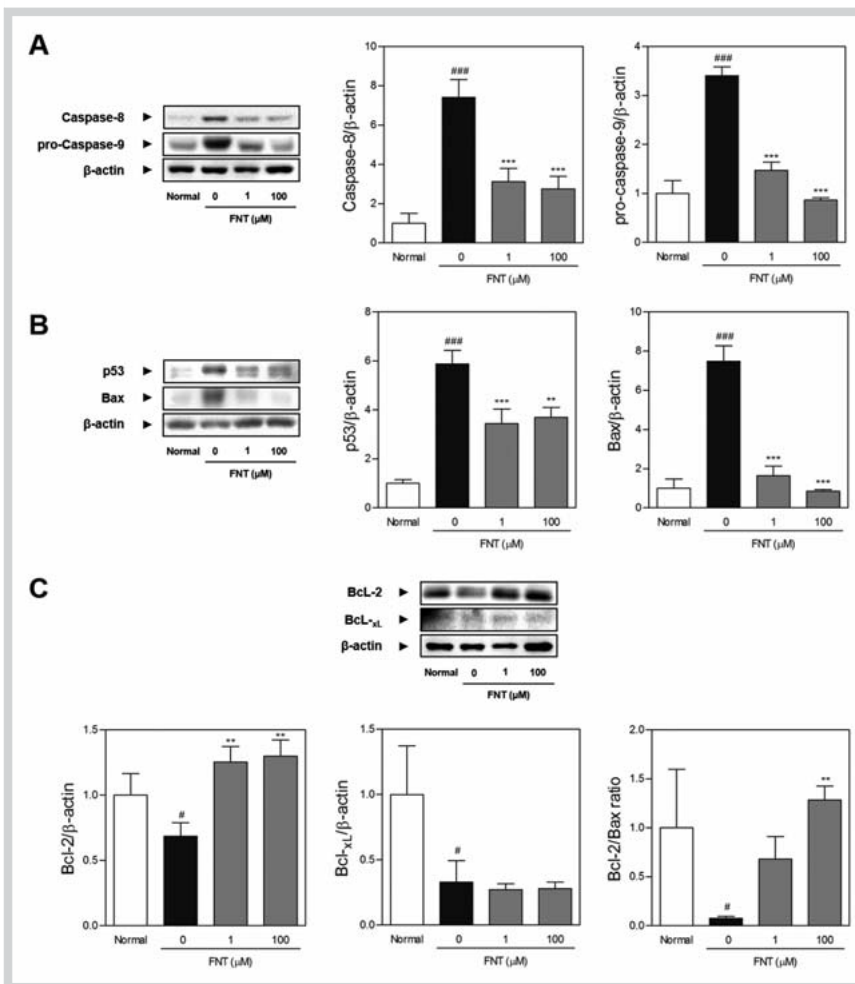


Fig. 2 The effects of formononetin on activation of caspase-8 and procaspase-9 (A), p53 and Bax (B), and Bcl-2, Bcl-xL and Bcl-2/Bax ratio (C). Data shown are mean \pm S. E. M. Mean values were significantly different for the following comparisons. *Marks significant differences as indicated by brackets ($p < 0.05$).

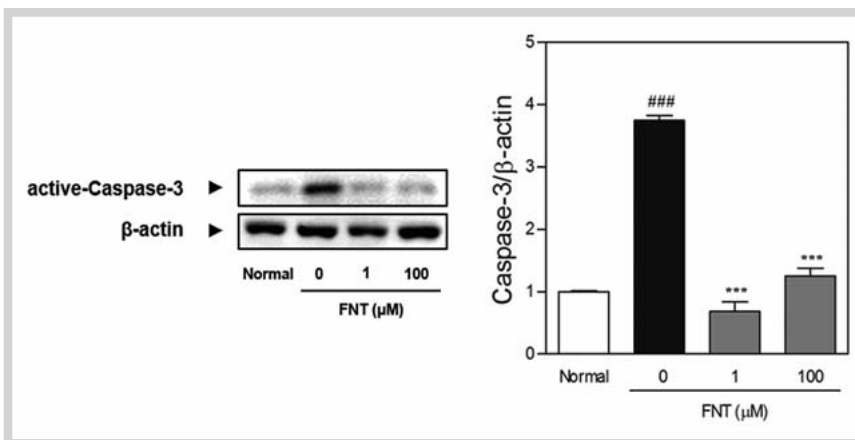


Fig. 3 The effects of formononetin on activation of active-caspase-3. Data shown are mean \pm S. E. M. Mean values were significantly different for the following comparisons. *Marks significant differences as indicated by brackets ($p < 0.05$).

tor p53 may serve as an inducer of pro-apoptotic Bax. It is crucial for the development of apoptosis in the hair follicles [28,29]. We showed that formononetin substantially inhibited the expression of p53.

Our findings demonstrate that the hair regrowth effects of formononetin are associated with both the Fas/Fas L-mediated extrinsic pathway, and the mitochondrial mediated intrinsic pathway, unlike conventional drugs such as finasteride and minoxidil. The use of formononetin may offer a possible alternative treatment for hair diseases. Future researches, including concentra-

tions of treatment, structure, percutaneous absorption and clinical studies in human, are necessary.

Materials and Methods

▼ Mice

Female C57BL/6 mice aged 7 weeks old in the telogen stage (pink color) of the hair cycle were purchased from Samtako Experimental Animal Center. The hair cycle of C57BL/6 mice with natu-

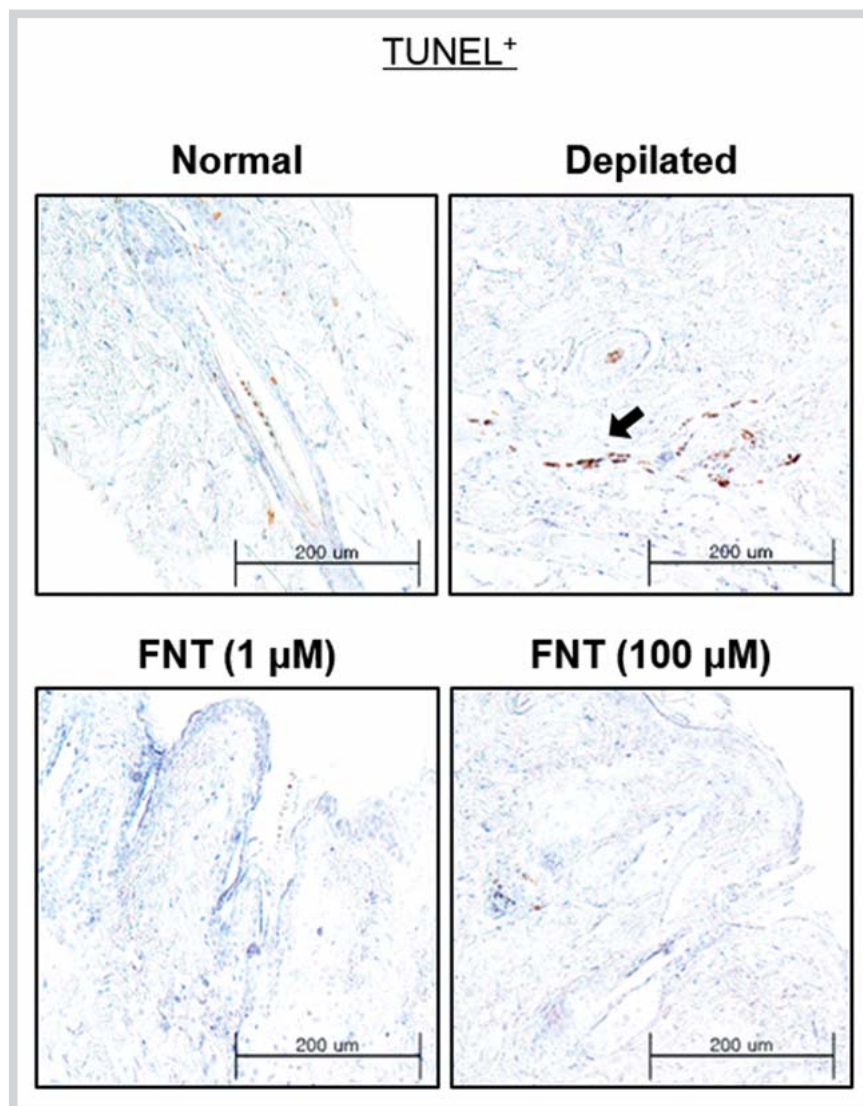


Fig. 4 The effects of formononetin on TUNEL-positive cells. The magnification is $\times 200$. Arrows indicate to TUNEL-positive cells. (Color figure available online only.)

ral hair loss is in the catagen phase, which recognized as gray skin color. The hair follicle regression by apoptosis in catagen occurs in naturally depilated mice.

The mice were housed in community cages with a 12 h light/dark cycle and fed standard mouse chow and water *ad libitum*. All experiments were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved on November 26, 2013 by Committee on Care and Use of Laboratory Animals of the Kyung Hee University (KHUASP(SE)-13-046).

Formononetin treatment

Animal treatment design was followed by the previous report [30, 31]. Formononetin ($\geq 99.0\%$; Sigma; cat. no. 47752) was dissolved in pure 100% methanol, vortexed and topically applied to depilated mice (FNT group) before the treatment. Animals were divided into four groups ($n = 7$): normal, depilated, FNT 1 μM and FNT 100 μM . The normal mice without depilation were assigned to normal group and the mice with natural hair loss were randomly assigned to Depilated, FNT 1 μM and FNT 100 μM . During the period of experiment, the depilated, FNT 1 μM and 100 μM groups were applied topically on the depilated back with 100 μL of vehicle (pure 100% methanol), 1 μM of formononetin

and 100 μM of formononetin, respectively. The normal group was not given any treatment. The treatment was continued for 2 weeks once daily. On day 15, all mice were photographed and sacrificed to take their dorsal skin. The dorsal skins were immediately fixed in formalin.

Histomorphometry

Hematoxylin and eosin staining was performed on paraffin-embedded 4 μm sections. Leica Application Suite (LAS) Microscope Software (Leica Microsystems Inc) was used to take photodocumentation. The magnifications used were $\times 100$ and $\times 400$.

Immunohistochemical detection of terminal deoxynucleotidyl transferase dUTP nick end labeling and caspase-3-positive cells

Paraffin-embedded tissue sections were rehydrated and treated with 3% H_2O_2 to reduce endogenous peroxidase. After blocking with 10% normal goat serum, the sections were incubated with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT) to detect TUNEL-positive cells, while the other sections were incubated with rabbit anti-caspase-3 antiserum (cell signaling) to detect caspase-3-positive cells. The reaction was revealed by 3,3'-diaminobenzidine (DAB; Sigma) substrate.

Leica Application Suite (LAS) Microscope Software (Leica Microsystems Inc) was used to take photodocumentation. The magnification used was $\times 200$.

Western blotting

Whole-tissue protein lysates were prepared with T-PER tissue protein extraction reagent (Pierce). Mouse dorsal skin tissues (500 mg, $n = 7$ per group) were homogenized in 2 mL extraction buffer with a protease inhibitor cocktail (Roche). The activation of β -actin, p53, caspases-3, -8, -9, B cell leukemia protein (Bcl)-2, Bcl_{xL} and Bcl-2-associated X protein (Bax) was investigated using the whole-tissue protein fraction. After incubation with each primary antibodies and anti-rabbit alkaline phosphatase-conjugated secondary antibody, visualized bands were detected using an enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia). Relative band densities were determined using a computerized densitometry system (Image J, NIH, Bethesda, MD, USA).

Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) and nonparametric tests. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

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Conflict of Interest

The authors declare no conflict of interest.

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