International Journal of Advanced Smart Convergence Vol.8 No.1 184-195 (2019)
http://dx.doi.org/10.7236/IJASC.2019.8.1.184

IJASC 19-1-23

Hair Growth Promoting Effect of Radish Crude Saponin Extract on Athymic Nude Mice

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Abstract

This study investigates the hair restoration efficacy of selected radish saponin extracts on nude mice. Nude mice genetically predisposed to pattern balding were used in this study. Our study revealed the underlying mechanism of stimulating hair growth in athymic nude mice by repair the nu/nu follicular keratin differentiation defect. Thus, the topical application of radish saponin may represent a novel strategy for the management and therapy of certain forms of alopecia. The term of hair density of PEE treated nude mice were significantly increase as compared with of control nude mice. Histological observation of skin sample showed no hair follicle or only distorted hair follicles were observed in the control samples, in contrast, by the PEE treatment groups showed a fully formed and increased the number of hair follicles up to three times higher than that of control group in terms of the number of hair follicles in nude mouse skin.PEE treated mice the number of BrdU-labeled keratinocytes per anagen follicle increased significantly, especially in the follicular bulbs and outer root sheath compared with the control mice. Moreover, PEE-treated nude mice also exhibited a significant increase in the number of BrdU-labeled epidermal keratinocyte proliferation.

Keywords: *Anagen , Radish crude saponin, Hair follicle, Athymic nude mice, BrdU*

1. Introduction

The hair follicle (HF) is the most prominent mini organ of the skin that remarkable for its dynamic structure [1-5]. The fundamental feature of hair biology is consisted with the production of a hair shaft (anagen), apoptosis-driven by regression (catagen), and relative resting (telogen). It undergoes repeated cycles of regression and regeneration throughout the lifetime of an organism. Every phase in the hair cycle is characterized by distinctive, strictly co-coordinated progression of tissue proliferation, differentiation, and

apoptosis, thus maintaining hairy phenotype of an organism [6, 7]. The nude mouse mutation has a pleotropic effect that causes the abnormal development of the skin, hair follicles, lack of fur coat and thymus [8, 9]. The most striking feature of nude mice (nu/nu) mutation has proven to be a complete lack of fur development after birth, has established a valuable and widely used biomedical tool since its discovery in 1966

Manuscript Received: Feb. 28, 2019 / Revised: Mar. 7, 2019 / Accepted: Mar. 13, 2019
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[8]. The transcription factor Foxn1 (Forkhead box formerly called Whn and Hfh11) was identified as the product of the nude gene on mouse chromosome 11 [9-11] which is now referred to as $Foxn1^{nu}$ [8] and also been detected in humans located on chromosome 17, exhibiting nude mouse-like phenotype [9, 13-15]. Although the nude mouse phenotype appears hairless at the skin but its dermis contains a considerable number of active hair follicles. However, follicles are aberrant and underdeveloped [8, 16] due to absence of functional Foxn1 and other inherited deformity related to keratin gene expression [17]. The impaired differentiation of nude follicle exhibit structural imperfections of the cortex, hair cuticle, and inner root sheath [18]. Resulting, hair shafts bend and coil at the stage of sebaceous gland and failed to penetrate the epidermis, thus makes the nude mice lack of external fur coat [19]. Various research groups has taken into account nude mouse as model for hair biology and widely reported their evaluation of synthetic compounds. Like as CsA [16, 20-24], KGF [25], AS101 [26], and PKC inhibitors, calcitriols and their related compounds used as potential therapeutic tools but most having adverse side effects on human health. Natural products either from pure compounds or standardized extract provide enormous opportunities to discover new therapeutic against the synthetic drugs. Chemically synthesized drugs are known as common for adverse side effects, so research turned on the ethnopharmacognosy. Course of investigation was carried out on nude mice skin to know the effect of natural extract of Radish crude saponin and its response on the distorted hair follicle. Nude mouse as widely used model in different areas of research like as immunological, dermatological, cosmetic, oncological, and transplantation research, particularly because of their defect in allo-or xenotransplant rejection [27,28]. The responsible nude gene 'nu' was shown to be of recessive, autosomal character [23]. This nude gene mutation may provide an ideal model for the study of various forms of human hair loss disorders involving alopecia, sebaceous gland hyperplasia, hyperkeratosis and defective differentiation of the epidermis [24].

Thefore the Radish saponin is well known to have a numerous number of therapeutic effects analgesic, anti-inflammatory, anti hepatitis C virus, antitumor and even reported to posses immunomodulatory activities. In oder to this study investigates the hair restoration efficacy of selected radish saponin extracts on nude mice.

2. Experiment Materials and Methods

2.1 Heat Treatment of Radish and Preparation of Extract

Radish was purchased from an agricultural and marine products wholesale market in Korea and the whole plant including leaves and peels were used after washing. A heat-treatment device (Jisco, Seoul, Korea) was used which was designed and constructed such that it could resist a pressure of 10 kg/cm^2 or higher. The whole radish sample was placed in an inner container which was then placed in an outer container containing a predetermined amount of water. Then, the radish sample was heated at a predetermined temperature for a predetermined time so as to prevent the sample from being carbonized by direct heat transfer. During the heat-treatment process the radish sample could be treated with steam. The heat-treatment temperature was set at 110, 120, 130, 140 and 150°C and the heat-treatment time was set at 6 hours.

2.2 Extraction and Fractionation of Radish saponin

The heat-treated radish sample was cooled and then ground using a grinder and a 10-fold volume (v/v) of distilled water was added there to followed by extraction for 14 to 16 hours. The extract was filtered and then freeze-dried and used. The sample was ground into powder (800g) and extracted three times with petroleum ether at 40°C for 4 hours under reflux then filtered and evaporated under vacuum to dryness to give the corresponding PEE. The resulting residue was then extracted three times with MeOH at 70 °C for 4 hours

then filtered and evaporated. The dried MeOH extract residue was suspended in distilled water and the resulting aqueous suspension was fractionated sequentially with hexane fraction (HeF) and *n*-butanol fraction (BuF) in a 1:1(v/v) ratio three times at room temperature. The resulting two fractions and remaining water fraction (WaF) were evaporated under vacuum to dryness.

2.3 Experimental animals

Athymic male nude (nu/nu) mice Balb/c origin at 7 weeks of age were purchased from Dae-Han Biolink (Eumsung, Korea). They were kept in autoclaved cages with filter bonnets in a laminar flow unit under 12-hour light and 12-hour dark periods at 24 ± 2°C in humidified atmosphere and were fed sterilized food and distilled water. Experiments were performed in Animal Center under aseptic conditions in accordance with approved institutional protocols by the Institutional Animal Care and Use Committee (IACUC).

2.4 Administration of PEE and fractions of Radish crude saponin

Mice were divided in to six groups; five males were allocated to each of six groups. While animal in group-1 received 0.4 ml of vehicle mixture containing propylene glycol (67% v/v), Ethanol (30%, v/v) and dimethyl sulfoxide (DMSO-3%, v/v) (Sigma, Mo, USA), animals in groups 2 received 5% Minoxidil 0.2ml). Group-3, group-4, group-5 and group-6 received 5% solution of PEE, HeF, BuF and WaF of radish crude saponin respectively. Topical application was performed once per day on the back of nude mice skin for 20 consecutive days.

2.5 Evaluation of hair coverage area

Mice were evaluated for hair coverage area by giving them a score of 0 to 8 as described in Table 1 (Suppl. Figure 1). Hair scores were taken on Day 0, 5, 7, 10, 16 and 20 by the three independent observers blinded to the treatment groups.

2.6 Evaluation of hair density

After daily treatment digital image were randomly taken on experimental Day 8 and 16 from each mouse in 35mm^2 area of interscapular region of the skin. The change of hair density were evaluated by analyzing the image (×200 magnification, actual area 35mm^2) using KONG, Bom-Viewer Plus (Seoul, Korea). Two people not familiar with the study quantified the hair density.

2.7 Histologic assessment of hair growth

Skin samples were fixed in 10% neutral buffered formalin for histological analysis. Paraffin-embedded 4μm sections were stained with hematoxylin and eosin (H&E). The nude mice HF morphology and the structure were evaluated microscopically in the H&E-stained sections of dorsal skin at a magnification ×1000. Five fields per section (×100 magnification) were considered for counting the number of dermal and subcutaneous hair follicle with respect to the total number of hair follicle. Histopathological processing and digital photomicrographs were taken using Leica application suite, Version 4.0.0, Leica Microystems (Switzerland) Limited.

2.8 Assessment of Keratinocyte Proliferation with Anti-BrdU

Keratinocyte proliferation was measured by intraperitoneal injection of BrdU(50 mg/kg body weight; Sigma Chemical Co., St. Louis, MO) at 1 hour before the mice were sacrificed. Dorsal skin from both treatment and control animals were collected on experimental day 16 and fixed with 4% paraformaldehyde,

dehydrated, and embedded in paraffin. After sectioning, the slides were dewaxed and denatured in 1.5 mol/L of HCl for 30 minutes and neutralized with phosphate-buffered saline for 1 hour. BrdU incorporation was detected by IHC staining of paraffin embedded sections with mouse anti-BrdU primary antibody (1:200) in a moist chamber, at room temperature for 3 hour. (Santa cruz Biotechnology, Santa cruz, CA, USA). After washing three times, incubated with secondary antibodies for 15 min. Skin sections that were not incubated with primary antibodies were used as a negative control. Assessment of follicular and epidermal keratinocyte and sebaceous gland epithelial cell BrdU labeling was done by an observer blinded to the treatment groups using the original magnification, x400.

2.9 Statistical analysis

The experimental data were expressed as mean ± standard error mean (SEM). Student's t-test or One-way ANOVA was used for assessment of significance between treatments. Statistical analysis was performed using SAS 9.2. A value of $P < 0.05$ was regarded as statistically significant.

3. Result and Discussion

3.1 Macroscopic observation of hair changing pattern on nude mice:

The ability of the hair changing pattern was documented after continuous topical application to athymic nude mice for 20 consecutive days. The effect of PEE of radish saponin was evident as early as 8 days after commencing treatment and became obvious on the dorsal body surface. Hair growth first appeared on the head then neck, shoulder area and the hip and consistently move to the caudal region of the tail on experimental day 16 (Figure 1B). On the other hand, control mice exhibiting relatively sparse and shorter hair and were roughly distributed abortive boundaries of anagen hair follicle on different region of the body noticeably corresponds to the 'wave like pattern' of nude mouse hair phenotype (Figure 1A). Eventually, control mouse became completely nude on experimental day 16 (Figure 1A & C), while PEE induced a distinct smother, thicker hair follicle and having the dense with most marked hair coverage on day 16 (Figure 1B & D).

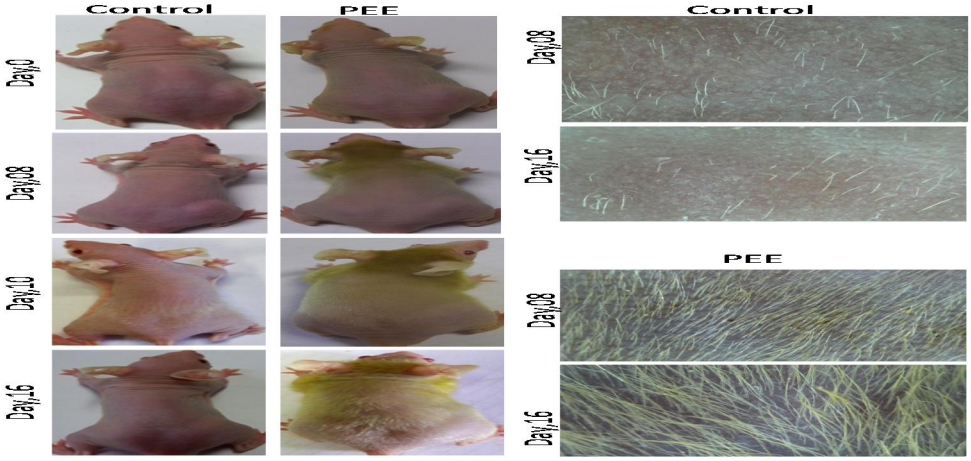

Figure 1. Stimulation of hair growth by PEE of radish crude saponin in nude mice was administered for 16 consecutive days. A) Vehicle (control) treated nude mice skin on Days 0, 08, 10, 16. Control mouse exhibiting nearly complete alopecia on Day 16. B) PEE treated nude mice skin on Days 0, 08, 10, 16 respectively, while PEE of radish crude saponin induced Profound effect is evident and having the dense hair coat with most hair coverage on Day 16. C) Skin surface reveals that the only a few hair shaft emerging from skin surface and leads to bending in control nude mice on Day 16 (arrow head). D)

PEE fraction has a normal dense hair coat on Day 16. Four mice /group were evaluated for each treatment group. Digital image were taken from skin surface of vehicle (C) and PEE (D) treated nude mice on Day 8 and 16 by KONG, Bom-Viewer Plus, 80X magnification lens.

3.2 Evaluation of hair coverage area

The stimulating effect of hair coverage area of nude mice were evaluated as they received specific concentration of PEE, HeF, BuF, WaF versus vehicle and or 5% Minoxidil. The time to appearance of hair varied from mice to mice. The effects of hair coverage area of all treated groups were precisely assessed for each mouse by giving them scores from a scale of 0–8 (Table 1). For example, a score of 8 represents the formation of the normal hair coat with full hair protrusion/extension above the dorsal skin surface. The maximum hair growth score was consistently increased in mice treated with the PEE than other groups from Day 8 to 16 (Figure 2).The HeF, BuF and WaF treated group had no effect or similar effect with vehicle control treated group regarding hair coverage area. While the PEE treated group consistently covered the maximum body coat during 16-18 days. In contrast, rapid hair loss of the Minoxidil and other treatment groups turns to baseline score before achieving maximal hair coverage.

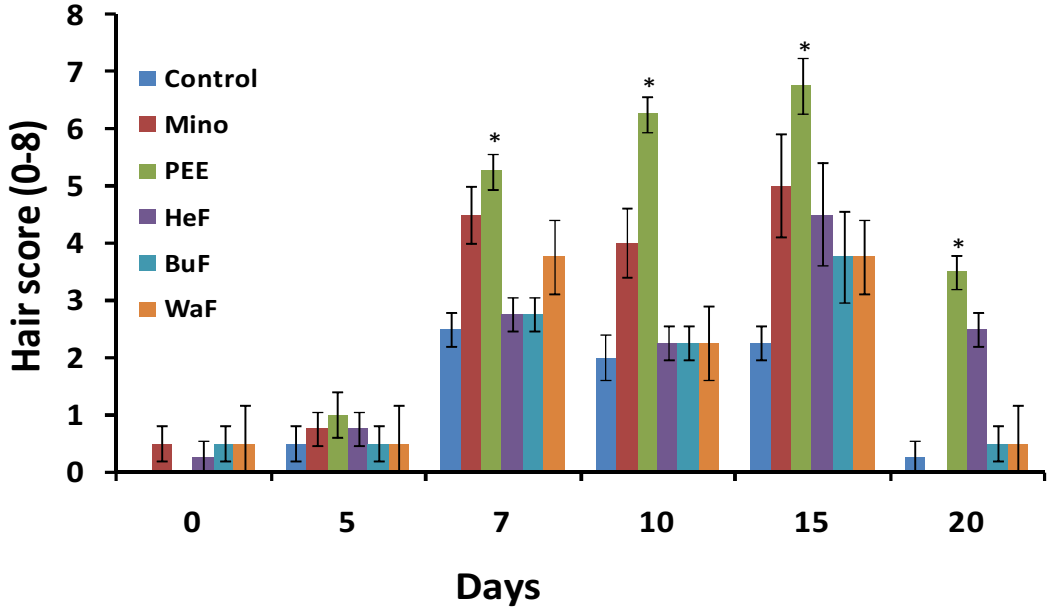

Figure 2. Monitoring of hair coverage area in athymic nude mice. Mice were topically treated with PEE, HeF, BuF, WaF versus vehicle control and or 5% Minoxidil for 20 consecutive days. Hair coverage area of different treatment groups on nude mice were evaluated by hair monitoring scale as illustrated in Table 1. All experimental groups were statistically analyzed for differences of hair coverage by the analysis of variance at 1% level of significance ($p < 0.01$).

3.3 Determination of hair density

Hair density was measured by analyzing the digital image that was taken from each mouse on experimental day 8 and 16. Pelage change/ the change of hair density in same region (2 cm diameter area) of interscapular skin were evaluated. The date of the pick hair growth was identified by the image showing the maximum hair density. Two person not familiar with the study quantified hair growth. The term of hair density of PEE treated nude mice (statistical values of PE treated mice) were significantly increase as

compared with of control nude mice (values of control mice). There was no difference among HeF, BuF, WaF and control nude mice. This similar finding was also observed for hair density (Figure 4), while EE fractions had lowest effect on sustaining hair density. PEE, HeF, BuF, WaF versus vehicle control and or 5% Minoxidil.

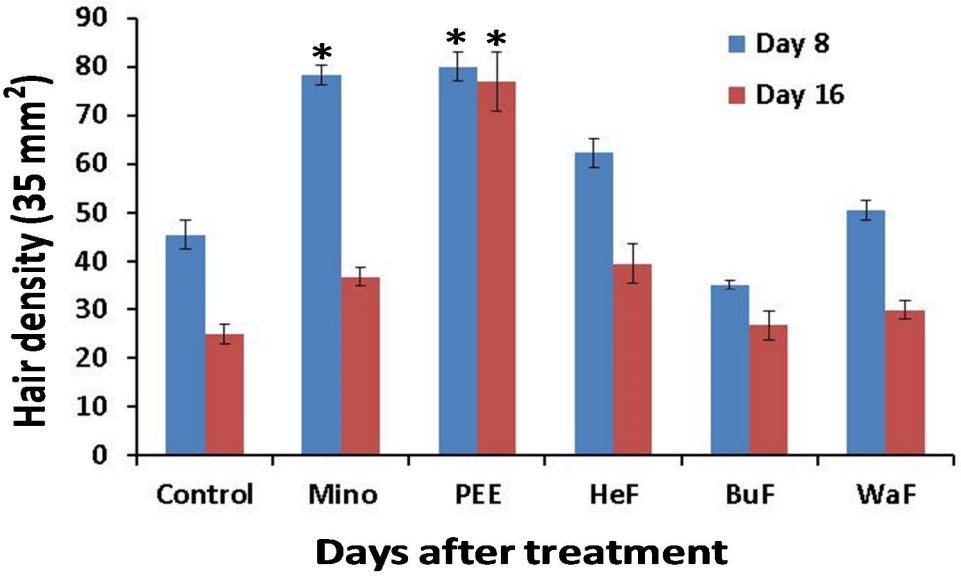

Figure 3. Hair density in athymic nude mice on experimental day 8 and day 16, treated for 20 consecutive days with PEE, HeF, BuF, WaF versus vehicle control and or 5% Minoxidil. All experimental groups were statistically analyzed for differences in hair density by the analysis of variance at 1% level of significance; PEE induces a statistically significant increase in hair density. Digital image were taken on day 8 and 16 by KONG, Bom-Viewer Plus (Seoul, Korea) with a 80X and 200X magnification lens.

3.4 Histological investigation of the skin of nude mouse

The skin specimens of control nude mice exposed with numerous dystrophic hair follicles, which contained fragmented cystically dilated by ketinaceous debris. At the level of sebaceous gland the hair shaft became distorted small and curly that fails to penetrate the epidermis (Figure 4A). However, those hair shafts that do penetrate the epidermis are heavily twisted and frequently fracture before achieving a substantial length. Nude mouse hair shaft twist and coil within the follicular infundibulam exactly represent in (Figure 4B, C), that hair fiber suffer from an abnormal keratinization. On the other hand, PEE treated nude mouse skin had relatively normal follicles containing well differentiated and unfragmented straight hair shafts which continued through the follicular ostia to the skin (Figure 4G). The structure of hair follicle of inner root sheath and hair shaft exhibit abnormalities, the most striking of which is the cuticle of the hair follicle was either discontinuous or, more often, totally absent (Figure 4D). In contrast the hair follicle of PEE treated mice were regularly formed and intact coated by a clearly discernible hair cuticle (Figure 4H) Furthermore, PEE treated group showed remarkable increase ($P<0.01$) of the number of hair follicles up to three times higher than that of control group (Table 1). On the other hand, hair follicles are histologically identical to follicles to late anagen of cycling hair.

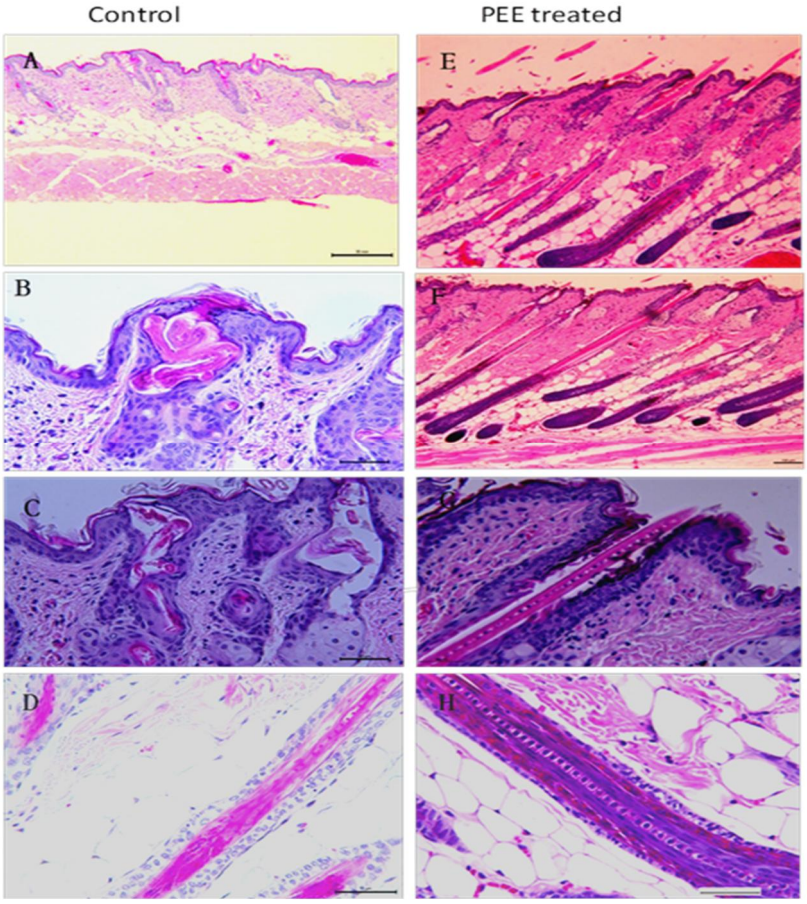

Figure 4. H&E- stained section of the skin from control and PEE treated athymic nude mice. A) Cystically dilated hair follicles filled with fragmented hair shafts and keratinized debris (arrows) that are normally present in nude mouse skin. E) PEE treated skin exhibits prominent follicular hypertrophy (arrows) as well as moderate sebaceous gland hypertrophy (arrowheads) with a normalization of follicular morphology and the presence of hair shafts above the skin's surface (arrows and inset).Note that PEE treated HFs were uniformly in late anagen phase of the hair cycle. D) Vehicle treated nude follicle, cortex formation is severely injured: IRS: inner root sheath, ORS: outer root sheath. F) Skin from PEE treated hair shaft regularly formed, intact and coated by a clearly discernible hair cuticle. (B, C)Skin of control nude mice shows hair shaft twist and coil at the level of sebaceous gland (B, C). (G)PEE treated mouse shows well differentiated straight hair shaft penetrating the skin surface shows no substantial difference with that of wild type. For further explanation, see text; RS: inner root sheath, ORS: outer root sheath, M: medulla, C: cortex, SG: sebaceous gland, HF: hair follicle, OST: Osteum. Scale bars: A, B = 100μm; C, D, E, F = 50μm.

3.5 Number of hair follicle

The effect of E. alba extract on hair follicles in the dorsum skin of the nude mice followed the effect of topical administration is shown in Figure 5B and Table 1. Histological observation of skin sample showed no hair follicle or only distorted hair follicles were observed in the control samples (Figure 5A), in contrast, by the PE treatment groups showed a fully formed and increased (Figure 5B) the number of hair follicles

(P<0.01) up to three times higher than that of control group in terms of the number of hair follicles in nude mouse skin (Table 1).

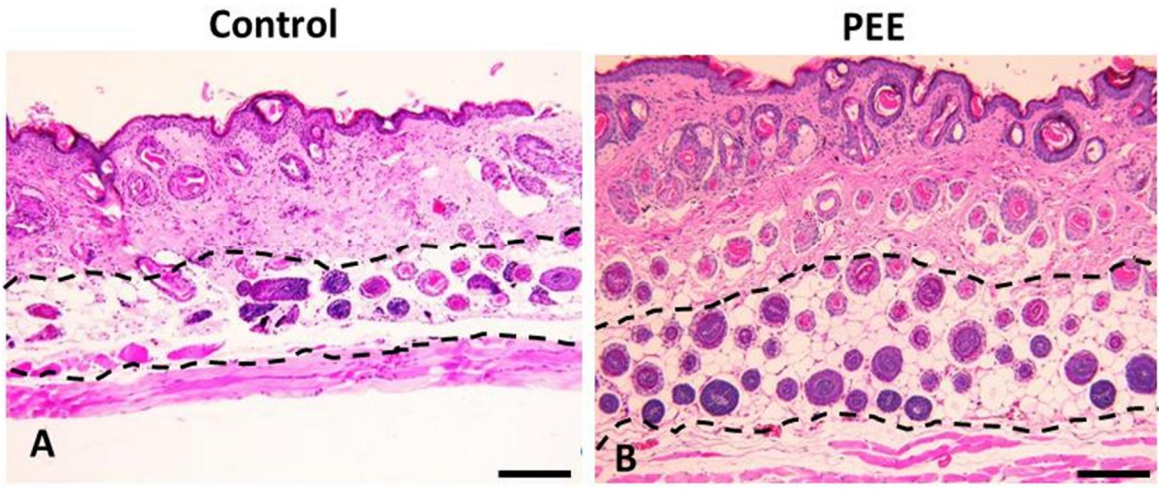

Figure 5. Number of hair follicle varies remarkably between a skin section from a control nude nouse with that of PE treated mice on day 16 (A and B). A) Control mouse with abortive and less number of hair follicle. B) Test nude mouse reveal normal and increase in number of hair follicles in the dorsal skin of PEE treated mice. The sections were stained with hematoxylin and eosin (original magnification, X100).

Table 1. Effect of PE extraction of radish crude saponin on follicle count in nude mouse skin.

Treatment	Hair follicles in Dermis layer	Hair follicles in Subcutis layer
Control	12.4 ± 0.8	22.6 ± 2.4
Minoxidil	18.6 ± 1.6**	20.4 ± 1.5
PEE	26.4 ± 0.81***	50.8 ± 1.1***

Values are mean (n=10) ± SD; **P < 0.01;*P < 0.001 compared with the vehicle-treated control. H & E stained histopathological data on hair growth anagen phase in the groups treated with PPE of radish saponin Statistical analysis was done using SAS version 9.1.3.**

3.6 Stimulates Proliferation of Follicular and Sebaceous Gland Keratinocytes

To quantify the difference in follicular keratinocyte proliferation rate we labeled the proliferating cells with 5-bromodeoxyuridine (BrdU) in vivo on Day 16 and subsequently stained them with an antibody to BrdU. Our results showed that PEE treated mice the number of BrdU-labeled keratinocytes per anagen follicle increased significantly, especially in the follicular bulbs and outer root sheath compared with the control mice. The mean number of BrdU positive cells per anagen follicle was 27 ± 4 in radish saponin PEE-treated mice as compared with 13 ± 4 in control mice hair follicle (data not shown). This increase was statistically significant (P<0.01). Moreover, PEE-treated nude mice also exhibited a significant increase in the number of BrdU-labeled epidermal keratinocyte proliferation. (Figure 6, Table 2).

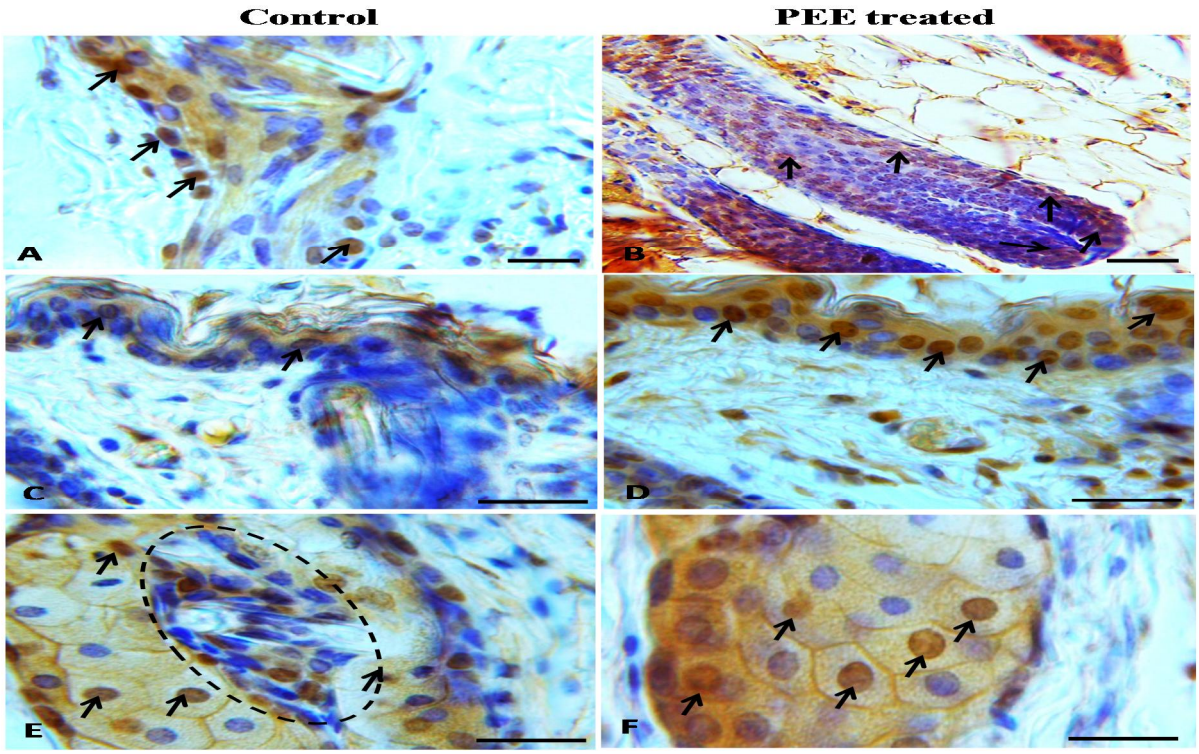

Figure 6. BrdU Immunohistochemistry for keratinocytes proliferation in control (Figure A, B, C) and PEE treated (Figure D, E, F) athymic nude mice skin. BrdU positive cells were detected in both the hair matrix and the outer root sheath (arrows). Arrow indicates follicular keratinocytes of the outer root sheath of hair bulb D: A moderate increase in BrdU-labeled follicular keratinocytes, particularly within follicular bulbs and outer root sheats (filled arrowheads), as well as a slight increase in BrdU-labeled peripheral epithelial cells in sebaceous glands (open arrowhead) in PEE treated nude mouse skin versus control nude mouse skin (C). Scale bar in A, 100 µm.

Table 2. Proliferation of BrdU positive keratinosites in PEE of radish saponin treated Nude Mice.

Treatment	keratinocytes per follicle	Epidermal keratinocyte per field	Epithelial cells per sebaceous gland
Control	14.9± 1.7	6.4 ± 1.6	8.4 ± 0.9
PEE	31.2±2.0***	22.2 ± 1.6***	14.2 ± 1.0**

Data are expressed as the mean (n=9) ± S.D. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control.
SAS Data (number of hair follicle in subcutis layer in 2nd hair cycle

4. Conclusion

This study was conducted to investigate the effect of radish saponin extract on the Radish saponin is well known to have a numerous number of therapeutic effects analgesic, anti-inflammatory, anti hepatitis C virus, antitumor and even reported to posses immunomodulatory activities. Taken together this information careful investigation was carried out on nude mouse skin driven by inherited hair follicular abnormalities and applied trustworthy hair natural stimuli agent Radish saponin. The unique findings reported here might be useful for better control of regeneration of human hair loss as well as could be used as alternative to the synthetic drugs. This study investigates the hair restoration efficacy of selected radish saponin extracts on nude mice. Learning about the normal development and synchronized cycling of HF between the developmental stages (anagen, catagen and telogen) is one of the key challenges for hair research. Most of our current knowledge of the substances which modulate hair growth in humans is derived from clinical observation and studies of mice have also been used to identify events associated with hair-follicle cycling. In genetically predisposed nude mice sparsely distributed HFs are barely visible on the nude skin. Moreover, progressive shortening of successive anagen cycles also leads to excessive shedding of HFs. These changes in hair growth and development stages are crucial because active HFs are still present and cycling, even in the skin of bald scalps or mutant nude skin. In this study, we investigated the hair-growth-promoting effect of topical application of radish crude saponin on nude-mouse skin. Plants producing numerous bioactive compounds and their fruitful application may promote hair regrowth act by up-regulating hair development. In this context, natural products are being paid more attention by researchers because there is a thriving clinical therapy that has already been proved even in the form of only a crude preparation. The mouse mutant “nude” is hairless, as described by Flanagan (1966) and athymic (Pantelouris, 1968). The hair defect of the nu/nu mutant is reported to be caused by imperfect keratinization in the hair shaft which causes the hair to break off at the skin level. The macroscopic appearance of hair growth of the control nude mice was documented as a few short, crippled, bent hair shafts emerging from the HFs. Very few follicles, of locally-variable density, occur in the dorsal skin ; and only during a short anagen growth phase. Here, we were able to observe an incomparable hair growth pattern in radish crude saponin treated mice with other treated groups. This consequence has raised the possibility that radish crude saponin might induce some signals that regulate the follicle to continue the growth phase of the anagen stage. Skin specimens of the radish crude saponin-treated mice were also found to have abundant BrdU-positive keratinocytes in their hair bulbs and outer root sheaths. One hypothesis is that temporary keratinization in the HF gives strength to hair shafts that rise above the skin surface. Moreover, in this study we have closely observed two generations of hair growth, as well as macroscopic analysis of hair growth parameters that included changing patterns of nude-mouse hair growth area of hair coverage and length of hair. The hair of radish crude saponin-treated mice achieved a substantial increase in length and became both thicker and smoother, whereas control hair shafts were transient, aberrant, short and irregular in shape, all suggesting a deeply impaired keratinization process.

Histological study of the different treatment groups showed that theradish crude saponin-treated group had an increased number of HFs in the deep subcutis, and had completely-developed HFs that corresponded to the anagen phase of the hair-growth cycle. Further detailed clinical trials and screening by chemical analysis will be necessary to identify the bioactive components in the extract. This knowledge will be used to prove the biological activity of the Radish crude saponin extract, and to show whether the whole extrac rather than individual components acts against alopecia.

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