***In Vivo* Hair Growth-Promoting Effect of Rice Bran Extract Prepared by Supercritical Carbon Dioxide Fluid**

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The potential hair growth-promoting activity of rice bran supercritical CO₂ extract (RB-SCE) and major components of RB-SCE, linoleic acid, policosanol, γ -oryzanol, and γ -tocotrienol, were evaluated with the histological morphology and mRNA expression levels of cell growth factors using real-time reverse transcriptase-polymerase chain reaction (PCR) in C57BL/6 mice. RB-SCE showed hair growth-promoting potential to a similar extent as 3% minoxidil, showing that the hair follicles were induced to be in the anagen stage. The numbers of the hair follicles were significantly increased. In addition, mRNA expression levels of vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and keratinocyte growth factor (KGF) were also significantly increased and that of transforming growth factor- β (TGF- β) decreased in RB-SCE-treated groups. Among the major components of RB-SCE, linoleic acid and γ -oryzanol induced the formation of hair follicles according to examination of histological morphology and mRNA expression levels of cell growth factors. In conclusion, our results demonstrate that RB-SCE, particularly linoleic acid and γ -oryzanol, promotes hair growth and suggests RB-SCE can be applied as hair loss treatment.

Key words rice bran supercritical CO₂ extract; hair growth-promoting activity; *in vivo*

Although hair loss is not a mortal disorder, it has a great impact on a person's self-respect, mental health, and overall quality of life. Approximately 50–60 hairs are normally lost per day, which does not have a noticeable effect on appearance; however, excess loss (>100) results in baldness. Androgenetic alopecia is the most common type of hair loss, affecting millions of both men and women.¹⁾ There are many causes of hair loss in men and women, including diseases, nutritional deficiency, aging, hormone imbalance, and stress. Androgenetic alopecia may occur as early as the teenage years, but typically begin in the later decades of life.^{2,3)} Hair loss affects at least half of all men by the age of 50 and up to 70% of 70-year-old men.⁴⁾

Topical minoxidil and oral finasteride approved by the Food and Drug Administration (U.S.A.) are typically used to treat androgenetic alopecia. Topical minoxidil, an adenosine triphosphate-sensitive potassium channel opener, was shown to be effective in 54% of treated patients as opposed to 34% in placebo control groups. However, there are significant adverse dermatological effects associated with minoxidil, including pruritis, dryness, scaling, local irritation, and dermatitis.^{5–8)} Oral finasteride, a competitive inhibitor of type-2 5- α -reductase, is known to increase hair growth in patients with male pattern baldness (androgenetic alopecia). It was reported that 48% of hair regrowth was observed in finasteride recipients in one year. Finasteride is generally well tolerated by patients, but some patients withdrew from treatment due to drug-related sexual disorders.^{9,10)}

Therefore, there remains a demand for highly effective pharmacotherapy for treating androgenetic alopecia with an excellent safety and efficacy profile. In the past several years, there have been numerous attempts to develop new agents

capable of preventing and/or treating pattern baldness.^{3,11,12)} Currently, natural extracts from several plants have been used for hair growth promotion, including *Asiasari radix*,¹³⁾ *Eclipta alba*,¹⁴⁾ essential oil of *Chamaecyparis obtuse*,¹⁵⁾ *Zizyphus jujube*,¹⁶⁾ and *Sophora flavescens*.¹²⁾

Rice (*Oryza sativa*) is one of the most important crops worldwide. It is a staple food for over half of the world's population with approximately 95% of rice produced in Asia and about 600 million tons of rice produced annually worldwide.^{17,18)} Rice bran is the major by-product of rice milling process and accounts for nearly 8% of milled rice. Between 20–30% of produced rice bran is used for oil production, while the remaining rice bran is discarded or used as livestock feed and fertilizer.¹⁹⁾ It has recently been reported that rice bran extract has various health beneficial effects, including antioxidant,¹⁹⁾ anticancer,²⁰⁾ and anti-hyperlipidemia activities.²¹⁾ In addition, rice bran extract shows 5- α -reductase inhibitory activities *in vitro* cell lines.²²⁾ However, it is unknown whether rice-bran extract is effective for treating hair loss *in vivo*.

Typically, rice bran oil is extracted using organic solvents, commonly hexane. Hexane is relatively simple and excellent for extracting nonpolar lipids.²³⁾ However, it is highly volatile and is considered toxic to animal and humans at relatively low concentrations. In addition, removing residual hexane is expensive and time consuming.^{23,24)} Supercritical fluid extraction has been introduced as an alternative one-step method conducted at low temperature for oil extraction. Extraction of oil at the critical point minimizes the thermal degradation of proteins, antioxidants, and other nutritionally valuable components. Supercritical carbon dioxide (SC-CO₂) has been used as a substitute for organic solvents during oil extraction, with advantages including that it is environmental friendly, non-toxic, nonflammable, and inexpensive. In addition, it can be easily removed from the final products.²⁵⁾

The authors declare no conflict of interest.

In the present study, we evaluated the potential hair growth-promoting activity of rice bran by comparing histological results and expression levels of cell growth factors from the skin of C57BL/6 mice treated with crude rice bran-supercritical carbon dioxide fluid extracts (RB-SCE) and its major components.

MATERIALS AND METHODS

Oryza sativa Bran Preparation and Materials Rice (*Oryza sativa* LINN. var. *japonica*; the Korean cultivars, Dongjin; Gramineae) used in this study were harvested in Gijang, Busan during the fall of 2011 and the rice bran was milled and provided by PN RICE Co., Ltd. (Kimhae, Gyeongsangnamdo, Korea) in March of 2012. Linoleic acid (LA) and authentic fatty acids for quantitative analysis were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Gamma-oryzanol (OZ) and policosanol (PS) were supplied from Oryza Oil and Fat Chemical Co. (Ichinomiya City, Japan) and Sinochem Qindao Co. (Qingdao, China), respectively. Gamma-tocotrienol (TT) was obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All chemicals and solvents used were of analytical grade.

Preparation of RB-SCE Supercritical CO₂ extractions of rice bran were performed in a semi-continuous flow-type apparatus with a 3-L extractor.²⁶⁾ Carbon dioxide was pumped into the extractor by a positive displacement controlled-volume metering-pump. A flow rate of 135-g CO₂/min was used for extraction. Pressure in the extractor was controlled using a back-pressure regulator. The extraction vessel was loosely packed with glass wool, and 1 kg of rice bran sample was added and distributed throughout the packing. A small plug of glass wool was then placed in the outlet end of the tube before closure to reduce entrainment. The extract was collected in a separator and chilled with ice by expanding the loaded solvent to ambient pressure. Extractions were performed at 32°C and 270 bar for 240 min. RB-SCE was stored at -80°C until use.

Analysis of Fatty Acids Fatty acid methyl ester mixtures (FAME) were prepared by esterification with alcoholic sulfuric acid reagent according to the International Union of Pure and Applied Chemistry (IUPAC) procedure.²⁷⁾ A GC-2010 series (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a flame ionization detector (FID) was used for gas chromatography (GC) analysis of methyl esters. Methyl esters were analyzed on an SPTM-2560 (Fused Silica Capillary Column, 100 m×0.25 mm×0.2 μm, Supelco, Bellefonte, PA, U.S.A.). The injection and detector temperatures were maintained at 225°C and 285°C, respectively. The flow rate of the carrier gas (helium) was 0.75 mL/min. The oven temperature was programmed to increase from 100°C to 240°C at the rate of 3°C/min after maintaining the temperature at 100°C for 4 min. FAME was identified using authentic standards, and peaks were quantified using digital integration according to the American Oil Chemists' Society official method Ce 1-62.²⁸⁾

Analysis of OZ, Tocols, Squalene, Policosanol, and Pytosterols The content of OZ was determined using spectrophotometry at 315 nm according to the method of Kim and Kim.²⁹⁾ To determine the tocopherols, phytosterols, policosanol, and squalene contents, 30 mL of ethanol was added into 0.5 g of RB-SCE with 5-mL 5% pyrogallol solution while heat-

ing with a reflux condenser. The solution was saponified with 1 mL aqueous 50% KOH solution for 5 min and mixed with 20 mL water and 30 mL diethyl ether. The mixture was extracted twice with 30 mL diethyl ether in a separator funnel. The pooled diethyl ether layer was washed 3 times with 20 mL distilled water, filtrated through anhydrous sodium sulfate, and evaporated at 30°C. After diluting with 10 mL chloroform and filtering through 0.45-μm FH membrane (Millipore, Billerica, MA, U.S.A.), the filtrate was analyzed for tocopherols using high-performance liquid chromatography (HPLC), while phytosterols, squalene, and policosanol were analyzed by GC. The HPLC apparatus (PU-1580; JASCO, Tokyo, Japan) was equipped with a Lichrospher Si-60 column (250×4.6 mm id; Merck Co., Darmstadt, Germany) and a fluorescence detector (FP-1520, JASCO) with excitation set at 298 nm and emission set at 325 nm. The isocratic mobile phase contained 1% 2-propanol in *n*-hexane. The flow rate was 1.0 mL/min. Tocopherol and tocotrienol peaks were evaluated by comparison external standards in the linear measuring ranges of 0.5–40 μg/mL. The GC (Varian 3800, Varian Inc., Walnut Creek, CA, U.S.A.) consisted of an SAC-5 fused silica capillary column (30 m×0.32 mm i.d.; Supelco) and flame-ionization detector. The column was held at 270°C for 1 min and programmed to 290°C for 20 min at a rate of 10°C/min. The carrier gas was helium, and the total gas flow rate was 20 mL/min. The injector and detector temperatures were 300°C and 320°C, respectively. Squalene, policosanol, and phytosterol peaks were identified by comparing retention times (RT) of each peak to those of pure standards.

Animals All animal procedures were approved by the Institutional Animal Care and Use Committee of Korea University. Five-week-old C57BL/6 mice (SLC, Shizuoka, Japan) were treated after acclimatizing to laboratory conditions for 1 week. Animals were allocated at 5 per polycarbonate cage in a temperature (20°C) and humidity (40–45%)-controlled room. The light:dark cycle was 12:12 h, and food (Samyang, Wonju, Korea) and water were supplied *ad libitum*.

To confirm the hair growth-promoting activity of RB-SCE, 6-week-old C57BL/6 mice were randomly divided into 3 groups as follows: negative control (NC; 10% ethanol as a vehicle), positive control (PC; 3% Minoxidil), and RB-SCE (3% in 10% ethanol) groups. To examine the hair growth-promoting activity of major components of RB-SCE, 6 groups were examined, including the NC group and groups treated with PC, LA (11.1 mg/mL), PS (0.03 mg/mL), OZ (0.22 mg/mL), and TT (0.0093 mg/mL). The concentrations of each component were corresponding amounts of LA, PS, OZ, and TT in 3% RB-SCE. Each 5 female mice per group were tested.

Determination of Hair Growth-Promoting Activity Hair growth-promoting activity of the RB-SCE was examined using the method reported by Roh *et al.*¹²⁾ with some modifications. Briefly, mouse hair was removed from a 2 cm×3 cm dorsal area of mice by carefully shaving with an electric clipper. Test materials (100 μL) were applied topically on the back skin of the mice once a day for 4 weeks. The hair growth-promoting activity of the substances was evaluated as darkening of the dorsal skin, indicating that the hair follicles were in the anagen phase. Hair growth scoring was performed by 2 independent dermatologists who were unaware of treatment regimen. The average of the each 2 scores was used as the

Table 1. Nucleotides Sequences of the Primers Used for PCR Amplification in This Study

Growth factor		Primer sequence
VEGF	Forward	ACS CGG TGG TGG AAG AAG AG
	Reverse	CAA GTC TCC TGG GGA CAG AA
IGF-1	Forward	TCA TGT CGT CTT CAC ACC TCT TCT
	Reverse	CCA CAC ACG AAC TGA AGA GCA T
KGF	Forward	ACG AGG CAA AGT GAA AGG GA
	Reverse	TGC CAC AAT TCC AAC TGC CA
TGF- β	Forward	GCG GCA GCT GTA CAT TGA CT
	Reverse	ACT GTG TGT CCA GGC TCC AA
GAPDH	Forward	CAA TGA ATA CGG CTA CAG CAA C
	Reverse	AGG GAG ATG CTC AGT GTT GG

hair growth index. Hair growth was measured once per week for 4 weeks by assigning a hair growth score as follows: score 0=no growth observed; 1=up to 20% growth; 2=20–40% growth; 3=40–60% growth; 4=60–80% growth; and 5=80% to full growth observed. Digital images of total hair growth on 4 weeks were obtained using Nikon Cool Pix P100 (Tokyo, Japan).

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) Total RNA was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD, U.S.A.) and the cDNA was synthesized by a reverse transcription reaction using the RNA PCR kit (Applied Biosystems, Roche Inc., Foster City, CA, U.S.A.) in a 20 μ L mixture containing 1 μ g RNA, 50 mM KCl, 10 mM Tris-HCl, 5 mM MgCl₂, 1 mM of each dNTPs, oligo(dT) primers, 20 U of RNase inhibitor, and 50 U of MuLV reverse transcriptase. Nucleotide sequences of the primers for vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), transforming growth factor- β (TGF- β), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in Table 1. The reaction mixture was incubated for 60 min at 42°C, and then heated at 90°C for 7 min in a thermocycler (GeneAmp PCR system 9600, PerkinElmer, Inc., Roche Molecular System, Waltham, MA, U.S.A.). Real-time PCR was performed using a Lightcycler instrument using FastStart DNA Master SYBR Green I PCR kit (Roche, Basel, Switzerland). Quantification of VEGF, IGF-1, KGF, and TGF- β mRNA expression was corrected by GAPDH.

Histological Analysis of Hair Follicles The test materials were applied topically on the lower side of dorsal skin of the mice once per day for 4 weeks. After week 4, all mice were sacrificed. Their dorsal skins were removed and fixed in 4% formaldehyde solution and embedded in paraffin. The fragments were sliced into transverse sections for determination of hair follicle count. In order to minimize the variation of histopathologic evaluation, the biopsy sites were set as the center of treated areas. The 3- μ m sections were stained with hematoxylin-eosin and toluidine blue and examined under a light microscope (Olympus, Tokyo, Japan).

Statistical Analysis Analysis of variance (ANOVA) and Tukey HSD *post-hoc* tests were performed for statistical analysis of data using SPSS (version 12.00, SPSS Inc., Chicago, IL, U.S.A.). A value of $p < 0.05$ was considered statistically

Fig. 1. Macroscopic Evaluation on Hair Growth Prompting Effects of RB-SCE on C57BL/6 Mice

(a): NC (negative control; 10% ethanol as a vehicle), (b): PC (positive control; 3% minoxidil), (c): 3% RB-SCE (3% rice bran ScCO₂ extract).

significant.

RESULTS

Hair Growth-Promoting Effect of RB-SCE To evaluate the hair growth-promoting activity of rice bran extract, 3% RB-SCE was applied on dorsal skin of C57BL/6 mice once per day for 4 weeks. As a negative control (NC) and positive control (PC), 10% ethanol and 3% minoxidil were topically applied, respectively. Figure 1 demonstrated the hair growth-promoting effects at 4 weeks for RB-SCE on C57BL/6 mice. In NC group, most mice showed only faint hair appearance after treatment for 4 weeks. In PC and RB-SCE treatment groups, mature hair was mostly occupied on the back of mice. The hair growth index of the RB-SCE group was compared with those of NC and PC groups as shown in Fig. 2. The hair growth index of RB-SCE group showed significantly higher values than that of the NC group, but similar values to the PC group after treatment for 4 weeks ($p = 0.002$), indicating the hair growth-promoting activity of RB-SCE.

Anagen Induction and Hair Restoration by RB-SCE on C57BL/6 Mice To evaluate the morphological structure of skin tissue, the histology of hair skin slices for each treatment group was tested. The results of histopathological examination at 4 weeks treatment showed no signs of irritation on treated area such as epidermal thickening or inflammatory cell infiltrations and so on (data not shown). The dorsal skin fragments of sacrificed mice after treatment for 4 weeks were stained with hematoxylin-eosin and toluidine blue (Fig. 3). The hair follicle formation of NC group was rarely observed. In contrast, the formation of hair follicles was observed in PC and RB-SCE treatment groups than that of the NC group. Particularly, in the skin of PC and RB-SCE groups, most hair follicles were fully induced and the hair root reached the deep subcutis, distinctly revealing growth of the inner and outer

root sheaths of hair according to toluidine blue staining.

To confirm changes in the hair growth cycle by applying the RB-SCE, the hair follicle formation in each group were compared (Fig. 4). The number of hair follicle in the PC and RB-SCE groups were 24 and 18 count/mm², respectively, which were significantly higher than 4 count/mm² in the NC group ($p=0.000$, Fig. 4).

Effect of RB-SCE on mRNA Levels of Growth Factors

To investigate the ability of RB-SCE to restore or inhibit hair loss, mRNA expression levels of VEGF, IGF-I, KGF, and TGF- β , hair growth related cytokines on the dorsal skin tissue of sacrificed mice after treatment for 4 weeks were

determined using real-time PCR (Fig. 5). Expression levels of VEGF and IGF-I of the mouse skin tissue treated with PC and RB-SCE were significantly higher than that of NC ($p=0.004$ for VEGF and $p=0.000$ for IGF-I). The expression level of KGF in the RB-SCE group was also significantly higher than that in the NC group. In addition, expression was significantly higher than that of the PC group ($p=0.000$). However, the level of TGF- β on the PC and RB-SCE groups was significantly lower than that of the NC group ($p=0.001$).

Based on these results, RB-SCE treatment on the dorsal skin of mice appeared to induce changes in the expression levels of growth factors, including VEGF, KGF, IGF-I, and

Fig. 2. Hair-Growth Index of C57BL/6 Mice after Topical Application of Rice Bran Extracts during 4 Weeks

Group 1: NC (negative control; 10% ethanol as a vehicle), group 2: PC (positive control; 3% minoxidil), group 3: 3% RB-SCE (3% rice bran scCO₂ extract).

Fig. 3. Hematoxylin-Eosin (Magnification $\times 40$: a, b, c. Magnification $\times 100$: d, e, f) and Toluidine Blue (Magnification $\times 40$: g, h, i. Magnification $\times 100$: j, k, l) Staining of the Skin Sections

The test materials were applied topically on the back skin of the mice once per day for 4 weeks. Negative control (10% ethanol as a vehicle; a, d, g, j), positive control (3% minoxidil; b, e, h, k), 3% RB-SCE (rice bran scCO₂ extract; c, f, i, l).

TGF- β genes, and the differentiation and proliferation of hair follicles, showing the ability of RB-SCE to promote hair growth.

Composition of RB-SCE To examine the hair growth-promoting activity of RB-SCE, the major composition of RB-SCE was analyzed (Table 2). RB-SCE was primarily composed of lipids. The amount of total triglyceride (TG) was 83.9g/100g RB-SCE, which was the major component. The TG of RB-SCE was mainly composed of 40.63% oleic acid, 38.42% linoleic acid, and 16.49% palmitic acid. Tocols,

policosanol, phytosterols, and squalene contents were determined to be 46.88, 92.33, 655.68, and 141.03 mg/100g RB-SCE, respectively. Particularly, γ -tocotrienol was 66.76% of total tocopherols. The amount of γ -oryzanol, which is known to be an anti-oxidant, was 0.7mg/100g RB-SCE. Based on their known biological activities, LA, PS, TT, and OZ were selected as candidate materials for hair growth-promoting activity.

Hair Growth-Promoting Effects of Major Components of RB-SCE To compare the hair growth-promoting activities of the major components of RB-SCE, LA, PS, OZ, and TT were applied to the dorsal skin of C57BL/6 mice at corresponding concentrations included in 3% RB-SCE once per day for 4 weeks. The hair growth indices for each component group were compared with those of NC and PC groups as shown in Fig. 6. LA- and OZ-treated groups exhibited outstanding hair growth-promoting potential, showing similar results with the PC group at treatment for 4 weeks ($p=0.0004$). However, PS- and TT-treated groups did not exhibit hair growth-promoting potential, showing similar results to the NC group.

Anagen Induction and Hair Restoration on Male C57BL/6 Mice by Major Components of RB-SCE The morphological structures of the tissue, obtained by examining transverse sections of the dorsal skin of each major component-treated group, are shown in Fig. 7. Dorsal skin fragments of sacrificed mice were stained with hematoxylin–eosin and toluidine blue. In the skin of the NC group, the formation of hair follicles was rarely observed and the follicles were cited in around boundary of dermis and adipose layer. In the PC,

Fig. 4. Comparison of Follicle Number in C57BL/6 Mice after Topical Application of Experimental Materials

Group 1: NC (negative control; 10% ethanol as a vehicle), group 2: PC (positive control; 3% minoxidil), group 3: 3% RB-SCE (3% rice bran seCO_2 extract) for 4 weeks. The asterisk indicates a statistically significant difference compared with negative control (***) $p<0.001$.

Fig. 5. mRNA Expression Levels of Growth Factors in the Skin of C57BL/6 Mice after Topical Application of Experimental Materials for 4 Weeks

(a) VEGF, (b) IGF-1, (c) KGF, and (d) TGF- β ; group 1: NC (negative control; 10% ethanol as a vehicle), group 2: PC (positive control; 3% minoxidil), group 3: 3% RB-SCE (3% rice bran seCO_2 extract) for 4 weeks. The asterisk indicates a statistically significant difference compared with control (** $p<0.01$).

Table 2. Concentrations and Compositions of RB-SCE Major Components

Components	Sub-components	Composition (%)	Total concentration
Fatty acids	Myristic acid	1.6	83.9 (g/100 g oil)
	Palmitic acid	16.3	
	Stearic acid	1.6	
	Oleic acid	41.1	
	Linoleic acid	36.4	
	Arachidic acid	0.6	
	γ -Linolenic acid	0.6	
	Linolenic acid	1.4	
	Arachidonic acid	0.4	
	Nervonic acid	0.1	
Policosanols	Tetra(C24)	3.5	92.33 (mg/100 g oil)
	Hexa(C26)	26.2	
	Hepta(C27)	5.8	
	Octa(C28)	2.4	
	Tria(C30)	62.0	
Tocols	α -Tocopherol	7.6	46.88 (mg/100 g oil)
	β -Tocopherol	5.9	
	γ -Tocopherol	10.0	
	δ -Tocopherol	0.9	
	α -Tocotrienol	5.5	
	γ -Tocotrienol	66.8	
	δ -Tocotrienol	3.3	
γ -Oryzanol			0.74 (g/100 g oil)
Phytosterols	Campesterol	20.1	655.68 (mg/100 g oil)
	Stigmasterol	15.1	
	β -Sitosterol	49.9	
	Cycloartenol	8.8	
	24-Methyl-cycloartanol	6.2	
Squalene			141.04 (mg/100 g oil)

LA, and OZ groups, most hair follicles were fully induced and the hair root reached the deep subcutis, clearly indicating the growth of inner and outer root sheath of hair. The epidermal cell differentiation into hair follicles and growth of hair follicles was observed in the skins of the PC and other groups by Toluidine blue staining. Although the follicles in the PS and TT groups were not fully induced to the extent of in the LA or OZ groups, downward growth of follicles from the dermis and increased follicles length were observed, which were different aspects from those of NC group.

To confirm the effect on hair growth cycle following application of major components of RB-SCE, the formation of hair follicles in each group were compared in the hematoxylin-eosin-stained sections (Fig. 8). The numbers of hair follicles of the PS and TT groups were 6 and 8 count/mm², respectively, which were not significantly different from those of the NC group. However, the number of hair follicles in the LA and OZ groups was 30 and 31 count/mm², respectively, which were significantly higher than that of PC group and NC group ($p=0.000$, Fig. 8).

Effect of RB-SCE Major Components on mRNA Expression Levels of Growth Factors To investigate the effect of the major components in RB-SCE as hair restoration or loss inhibition, the mRNA expression levels of VEGF, IGF-I, KGF, and TGF- β on the dorsal skin tissue of sacrificed mice 4 weeks after treatment were determined using real-time PCR (Fig. 9). The expression level of VEGF of the mouse skin tissue treated with PC and major components of RB-SCE was significantly higher than that of NC ($p=0.002$). The expression levels of IGF-I and KGF of the mouse skin tissue treated with PC and major components of RB-SCE were also significantly higher than that of NC ($p=0.000$ for IGF-I and 0.001 for KGF). In addition, in all tested group, the expression level of TGF- β was lower than that in the NC group ($p=0.006$).

Based on these results, the hair growth-promoting activity of RB-SCE likely resulted from changes in expression levels of growth factors, such as increased expression of VEGF,

Fig. 6. Hair Growth Index of C57BL/6 Mice after Topical Application of Experimental Materials during 4 Weeks

Group 1: NC (negative control), group 2: PC (positive control; 3% minoxidil), group 3: LA (linoleic acid; 11.1 mg/mL), group 4: PS (policosanols; 0.03 mg/mL), group 5: OZ (γ -oryzanol; 0.22 mg/mL), group 6: TT (γ -tocotrienol; 0.0093 mg/mL).

Fig. 7. Hematoxylin-Eosin (Magnification $\times 40$: a, b, c, d, e, f. Magnification $\times 100$: g, h, i, j, k, l) and Toluidine Blue (Magnification $\times 40$: m, n, o, p, q, r. Magnification $\times 100$: s, t, u, v, w, x) Staining of the Skin Sections

The test materials were applied topically on the back skin of the mice once per day for 4 weeks. Group 1: NC (negative control; a, g, m, s), group 2: PC (positive control; 3% minoxidil; f, l, r, x), group 3: LA (linoleic acid; 11.1 mg/mL; b, h, n, t), group 4: PS (policosanol; 0.03 mg/mL; c, i, o, u), group 5: OZ (γ -oryzanol; 0.22 mg/mL; d, j, p, v), group 6: TT (γ -tocotrienol; 0.0093 mg/mL; e, k, q, w).

Fig. 8. Comparisons of Follicle Number in C57BL/6 Mice after Topical Application of Experimental Materials

Group 1: NC (negative control), group 2: PC (positive control; 3% minoxidil), group 3: LA (linoleic acid; 11.1 mg/mL), group 4: PS (policosanol; 0.03 mg/mL), group 5: OZ (γ -oryzanol; 0.22 mg/mL), group 6: TT (γ -tocotrienol; 0.0093 mg/mL) for 4 weeks. The asterisk indicates a statistically significant difference compared with negative control (***) $p < 0.001$.

KGF, and IGF-I genes and decreased expression of TGF- β , induced from the major components LA, OZ, PS, and TT, and from the differentiation and proliferation of hair follicles induced by LA and OZ.

DISCUSSION

Generally, hair follicles are known to renew cyclically through 3 phases: anagen, catagen, and telogen. Hair follicles in each phase have distinct morphological characteristics. During anagen, the hair bulb is enlarged and it encloses the dermal papilla. The follicles show downward growth from the dermis *via* the panniculus adiposus to the panniculus carnosus.³⁰ Hair matrix cells proliferate and differentiate into daughter cells, which move upwards to form the inner root sheath and hair shaft. The length of hair follicle increases

continuously as the hair bulb enters the hypodermis. The skin becomes thicker and the number and size of hair follicles increase during this stage.³¹ During the catagen phase, follicles involute and skin thickness decreases. Through the telogen phase, both the follicles and the skin are at rest. When stem cells in hair follicles are activated, the follicle enters a new anagen phase and a new hair shaft is produced.

Various cytokines and growth factors play important roles in hair growth control. To promote hair growth and initiate anagen, it is essential that expression of factors maintaining anagen is increased, such as IGF-1, basic fibroblast growth factor (bFGF), KGF, and VEGF, while decreasing expression of cytokines promote apoptosis, such as TGF- β , and IL-1.^{32,33}

VEGF plays a central role in promoting angiogenesis as well as influencing diverse cell functions including cell survival, proliferation, and generation of nitric oxide and prostacyclin.³⁴ The expression of VEGF is related to the formation of blood vessels. VEGF mRNA expression during the hair cycle was variable, increasing during the anagen phase and then regressing during the catagen and telogen phases. IGF-1 is a peptide hormone that promotes the growth, survival, and differentiation of cells in various organ and tissues, including skin. IGF-1 is critically involved in promoting hair growth by regulating cellular proliferation and migration during hair follicle development.³⁵ KGF also has been shown to be expressed in the dermis and to regulate epidermal proliferation and differentiation *via* a paracrine mechanism, stimulating wound healing, and hair growth.³⁶ In contrast, TGF- β induces apoptosis in keratinocytes against cell death, indicating that TGF- β is involved in apoptosis-driven catagen development.³⁷

In this study, the shaved back skins of 7-week-old C57BL/6 mice were topically treated with RB-SCE for 4 weeks. Because the first cycle in mouse skin is synchronized, 6–7 week-old C57BL/6 mice are specifically known to be in the stable telogen phase. Thus, skin of 7 week-old C57BL/6 mice

Fig. 9. mRNA Expression Levels of Growth Factors in the Skin of C57BL/6 Mice after Topical Application of Experimental Materials for 4 Weeks

(a) VEGF, (b) IGF-1, (c) KGF, and (d) TGF- β ; Group 1: NC (negative control), group 2: PC (positive control; 3% minoxidil), group 3: LA (linoleic acid; 11.1 mg/mL), group 4: PS (policosanols; 0.03 mg/mL), group 5: OZ (γ -oryzanol; 0.22 mg/mL), group 6: TT (γ -tocotrienol; 0.0093 mg/mL). The asterisk indicates a statistically significant difference compared with control (** $p < 0.01$, *** $p < 0.001$).

was used for examining the effects of hair growth-promotion in hair follicle cycling.

The macroscopic and histological alterations in the skin of mice were evaluated for examining the effects of hair growth-promotion as a result of the treatments of RB-SCE. The growth rate of hair in RB-SCE treated group was higher than in NC group and similar with that of minoxidil group (Figs. 1, 2). In the histological evaluation, most hair follicles were fully induced and the hair root reached at deep subcutis, distinctly revealing the growing inner and outer root sheath of hair due to RB-SCE application (Figs. 3, 4). These results were similar to those of minoxidil. The action mode of minoxidil on the hair growth effect was not completely elucidated. However, mechanisms underlying hair growth stimulated by minoxidil have been reported. Otomo³⁸ proposed that minoxidil functions as a sulfonylurea receptor (SUR) activator and prolongs the anagen phase of hair follicles through by inducing cell growth factors such as VEGF, HGF, and IGF-1.

The mRNA expression levels of VEGF, IGF-I, and KGF following RB-SCE treatment were higher than those of the negative control group were. Particularly, the expression level of KGF by RB-SCE treatment was significantly higher than that of minoxidil (Fig. 5).

The present results showed that during the treatment with RB-SCE, an increase in the number of hair follicles, and increased expression levels of cell growth factors such as VEGF, HGF, and IGF-1, and decreased expression of TGF- β were confirmed, suggesting that RB-SCE may induce differentiation and proliferation of hair follicles through the expression of cell growth factors and resulting in the induction of the early anagen phase.

To confirm the main factors promoting hair growth in RB-SCE, the major components of RB-SCE were analyzed. The lipids of rice bran were comprised mainly of TG (80.6–86.0 wt%), free fatty acid (4.2–9.0 wt%), and phospholipids (5.5–6.7 wt%), while other components were also detected in minor proportions (0.2–2.1 wt%).³⁹ According to the results of Manosroi *et al.*,¹⁹ the raw rice bran oil contains both unsaturated and saturated fatty acids, in which palmitic acid is a major saturated fatty acid (C18:0, 12–26%, w/w, typically 18%, w/w). Unsaturated fatty acids primarily included oleic acid (C18:1, 35–46%, w/w, typically 42%, w/w) and linoleic acid (C18:2, 25–38%, w/w, typically 37%, w/w) with traces of γ -linolenic acid (C18:3, 0.4–3.8%, w/w). Rice bran is known to contain significant levels of tocopherols up to 300 mg/kg.⁴⁰ Rice bran also contains 3000 mg/kg OZ, which is a mixture of 10 ferulate esters of triterpene alcohol.⁴¹ In our study, the fatty acid profile of RB-SCE was similar to those stated in previous reports. However, the minor components of rice bran differed compared with those stated in previous reports, which was likely due to the difference in rice strains and extraction conditions used based on supercritical CO₂ extraction.

Among the main components, LA, OZ, PS, and TT were selected to examine the hair growth-promoting activity of RB-SCE. In particular, the unsaturated fatty acids, such as γ -LA, LA, and oleic acid, have been shown to have anti-hair loss activity by inhibiting the 5- α -reductase enzyme in androgen responsive organs.⁴² γ -Oryzanol has several important physiological effects, including hypocholesterolemic, anti-inflammatory, and antioxidant activities.⁴³ More than 50 studies indicate that policosanols decreases serum cholesterol, while other studies failed to reproduce this effect,⁴⁴ and tocotrienol possess po-

tent antioxidant activity.⁴⁵⁾

Upon examining the hair growth-promoting effects of the major components in RB-SCE, LA and OZ exhibited outstanding hair growth-promoting potential, showing similar results to that of 3% minoxidil based on macroscopic and histological evaluation (Fig. 6–8). Especially, in the LA and OZ groups, the number of hair follicles was markedly increased compared to the negative control group. In addition, mRNA expression levels of VEGF, IGF, and KGF in groups treated with LA and OZ were significantly higher than those of NC and mRNA expression levels of TGF- β were significantly lower than those of NC (Fig. 9).

And although PS and TT tested in this study were able to up-regulate the expression levels of VEGF, IGF-1, and KGF mRNA and to down-regulate expression level TGF- β mRNA, in terms of hair growth promoting actions, they were not able to positively affect the hair growth. This disagreement between histological evaluation and mRNA expression levels of growth factors in PS and TT treated groups may be able to explain with the concentration differences of treated materials. To confirm the major active components in RB-SCE, the corresponding concentrations of LA, OZ, PS, and TT included in 3% RB-SCE were applied to the dorsal skin of C57BL/6 mice, which were 11.1 mg/mL, 0.22 mg/mL, 0.03 mg/mL, and 0.0093 mg/mL, respectively. The concentrations of applied PS and TT were lower than that of LA and OZ, approximately from 10^{-1} folds to 10^{-3} folds. Accordingly, further studies are necessary to reconcile this disagreement between histological evaluation and mRNA levels in PS and TT treated groups, through adjusting the concentration levels of treatment components.

Nevertheless, the increase of hair index, the abundance of hair follicles in mice skin, and the induction of the change in expression levels of growth factors via RB-SCE, LA and OZ applications clearly supported that LA and OZ may act as the main factors for hair growth promoting and resulting earlier telogen-to-anagen conversion in RB-SCE.

A previous report showed that LA has strong hair growth-promoting effect *in vitro*.²²⁾ According to our results, LA and OZ showed outstanding hair growth-promoting effects. This is the first report that LA and OZ have hair growth-promoting effects *in vivo*.

In conclusion, this study provides potent evidence that RB-SCE, which contains LA and OZ, exhibited outstanding hair growth-promoting potential and suggests that these substances can be applied as hair loss treatments.

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