Phytomedicine, Vol. 7(6), pp. 529–536

http://www.urbanfischer.de/journals/phytomed

Phytomedicine

Investigation of topical application of procyanidin B-2 from apple to identify its potential use as a hair growing agent

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Summary

Procyanidin B-2 is a polyphenol compound we have identified in apple which acts as a hair-growing factor in the murine model both *in vitro* and *in vivo*. This report describes our investigation of the effects of 1 % procyanidin B-2 tonic on human hair growth after sequential use for 6 months. A double-blind clinical test involving a total of 29 subjects was performed. Nineteen men in the procyanidin B-2 group and 10 men in the placebo control group were subjected to analyses. No adverse side effects were observed in either group. The hair-growing effect was evaluated using a macrophotography technique combined with measurements of the hair diameter of clipped hairs. The increase in number of total hairs in the designated scalp area (0.5 cm square = 0.25 cm^2 area) of procyanidin B-2 group subjects after the 6-month trial was significantly greater than that of the placebo control group subjects (procyanidin B-2, 6.68 ± 5.53 (mean ± SD)/0.25 cm^2; placebo, 0.08 ± 4.56 (mean ± SD)/0.25 cm^2; $P < 0.005$, two-sample *t* test). The increase in number of terminal hairs, which are defined as hairs more than 60 μm in diameter, in the designated area (0.5 cm square = 0.25 cm^2 area) of the procyanidin B-2 group subjects after the 6-month trial was significantly greater than that of the placebo control group subjects (procyanidin B-2, 1.99 ± 2.58 (mean ± SD)/0.25 cm^2; placebo, –0.82 ± 3.40 (mean ± SD)/0.25 cm^2; $P < 0.02$, two-sample *t* test). Procyanidin B-2 therapy shows potential as a safe and promising cure for male pattern baldness.

Key words: androgenetic alopecia, condensed tannin, external application, *Malus pumila*, proanthocyanidins, scalp.

Introduction

Proanthocyanidins are a species of polyphenol that have a wide range of pharmacological effects (Haslam, 1996). They have been used as skin-protective cosmetics (Wayne, 1996) and as a treatment for capillary stabilization (Brasseur, 1989; Dartenuc et al., 1980). We have reported that procyanidin oligomers such as procyanidin B-2 (Fig. 1) possess growth-promoting activity in murine hair epithelial cells at a very high rate of 300 % relative to controls, and have also demonstrated that procyanidin oligomers stimulate anagen induction in the *in vivo* murine model at almost the same intensity as minoxidil (Takahashi et al., 1998; Takahashi et al., 1999a). We isolated procyanidin B-2 to a purity exceeding 94%(w/w) from apple juice and subjected it to a series of toxicological studies (Takahashi et al., 1999b). Our results confirm the safety of topical application of procyanidin B-2 to human skin. We report here on a clinical trial focusing on topical application of procyanidin B-2 of high purity to test whether it possesses a curing effect on male pattern baldness by means of assessment of change in hair density and terminal hair formation. The aims and objectives of this study were to identify any remedial effects on male pattern baldness and to investigate the desirability of pro-

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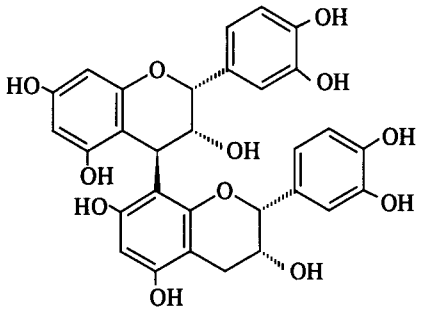

Fig. 1. Structure of procyanidin B-2 [epicatechin-(4β→8)-epicatechin].

ceeding to large-scale clinical trials of topical procyanidin B-2.

Materials and methods

Patients

To investigate the effects of topical application of procyanidin B-2 on the scalp and hair, a placebo-controlled clinical trial was performed at the company (Tsuchiura Plant, Kyowa Hakko Kogyo Co., Ibaraki, Japan) on volunteer employees. From 50 applicants, 30 volunteer subjects (30 to 57 years old, in good health) were chosen by pre-examination under the criteria that they showed male pattern baldness on the scalp, had no dermatological disorders other than male pattern baldness on the scalp, had no other diseases, and were not undergoing any medical treatment. The pattern of baldness was classified according to the Ogata scale (Ogata, 1953; Takashima et al., 1981) specific to Japanese males. They were divided into two groups using cards, assigned according to the degree of baldness, and then randomly divided into a 1 : 2 ratio (placebo : treatment) within each grade. The test was then started after confirming that there were no significant differences between the two groups as to background factors such as age or type and degree of baldness.

Study schedule

One group (20 men) was treated with 1 % procyanidin B-2 agent, and the other group (10 men) was treated with a placebo control. For 6 months, 1.8 ml of the test agent was applied to the subjects' affected area of the head twice a day, resulting in a daily dose of 30 mg of procyanidin B-2. No use of other hair care products except shampoos and rinses was permitted during the clinical trial. The tests were performed in double-blind fashion.

Determination of change in hair density

Before and after the test, hairs at a predetermined site (a round area 1 cm in diameter) of the subjects were clipped with small straight surgical scissors (Tsuji et al., 1994). The site was selected from the outskirts of the affected area on the vertex of each subject using a plastic template connected by a strut to the frame of a pair of eyeglasses. The hair-cutting sites were photographed using a camera (OM-4 Ti, Olympus Optical Co., Tokyo, Japan) fitted with a macro lens (Zuiko Auto-macro 20 mm, F2, Olympus Optical Co., Tokyo, Japan). The hairs in the photograph of this specific area (0.5 cm square = 0.25 cm^2 area on natural size) in a predetermined site were counted by three independent investigators three times each in double-blind fashion.

Determination of terminal hair formation

The diameter of the collected hairs was measured. The diameters of the bases of the hairs were measured using a micrograph-equipped microscope (BH-2, Olympus Optical Co., Tokyo, Japan) at a magnification of x 300 (Rushton et al., 1983). The analyses were all performed by investigators in double-blind fashion. Thus, the ratio of terminal hairs, which are defined as hairs more than 60 μm in diameter (Ishino et al., 1994), versus total hairs was determined. The number of terminal hairs in a designated area (in 0.5 cm square) was calculated from the total hair number (in 0.5 cm square) mentioned above and the ratio of terminal hair.

Table 1. Background factors.

Group	Number of subjects	Mean age	Patterns of baldness[1]		Degrees of baldness[2]		
			Type I	Type IV	Slight	Moderate	Severe
Placebo	10	48	6	4	2	6	2
PB2[3]	19	45	11	8	4	11	4

[1] According to the Ogata scale (Ogata, 1953; Takashima et al., 1981).
[2] Placed in three ranks: slight, moderate and severe.
[3] PB2: procyanidin B-2.

Table 2. Total and terminal hair growth in each subject.

Group	Case	Total hairs (in 0.25 cm^2)			Terminal hairs[1] (in 0.25 cm^2)		
		0 M[2]	6 M	Increase	0 M	6 M	Increase
Placebo	P1	37.0	37.3	0.3	2.24	3.18	0.94
	P2	49.0	50.4	1.4	9.56	10.83	1.27
	P3	34.0	37.0	3.0	25.77	23.19	–2.58
	P4	34.0	37.7	3.7	0	3.56	3.56
	P5	34.0	32.5	–1.5	7.56	7.31	–0.25
	P6	23.0	21.0	–2.0	16.35	13.77	–2.58
	P7	29.3	29.3	0	7.01	10.74	3.73
	P8	26.5	31.3	4.8	15.4	12.78	–2.62
	P9	35.3	37.7	2.4	13.24	5.69	–7.55
	P10	42.8	31.5	–11.3	4.76	2.67	–2.09
	Mean			0.08*			–0.82**
	SD			4.56			3.40
PB2[3]	V1	43.0	46.5	3.5	19.52	23.06	3.54
	V2	33.0	38.0	5.0	11.18	8.44	–2.74
	V3	31.7	39.0	7.3	7.13	13.0	5.87
	V4	29.3	47.6	18.3	0.85	3.97	3.12
	V5	39.8	54.3	14.5	0.73	2.26	1.53
	V6	37.3	44.0	6.7	10.28	7.16	–3.12
	V7	37.3	46.3	9.0	1.62	1.45	–0.17
	V8	30.7	30.0	–0.7	2.65	7.5	4.85
	V9	17.5	25.3	7.8	0	2.81	2.81
	V10	33.0	29.0	–4.0	3.93	7.04	3.11
	V11	36.0	42.0	6.0	5.0	11.69	6.69
	V12	42.0	46.5	4.5	12.13	12.35	0.22
	V13	39.0	47.0	8.0	3.04	3.84	0.8
	V14	26.0	37.4	11.4	17.33	21.28	3.95
	V15	21.0	22.0	1.0	13.43	15.81	2.38
	V16	29.0	36.3	7.3	0	1.2	1.2
	V17	19.5	28.0	8.5	5.32	8.35	3.03
	V18	29.7	29.0	–0.7	15.56	16.27	0.71
	V19	29.5	43.0	13.5	0	0	0
	Mean			6.68*			1.99**
	SD			5.53			2.58

[1] Terminal hairs: defined as > 60 μm in diameter.
[2] M: month(s).
[3] PB2: Procyanidin B-2.
* $P < 0.005$, two-sample t test.
** $P < 0.02$, two-sample t test.

Statistical analysis

The differences of total hair increase and terminal hair increase between the placebo and treatment groups were analyzed using the two-sample t test (Table 2). Total hair increase and terminal hair increase after the 6-month trial period compared with the baseline of every subject in each group were analyzed using the paired t test (Figs. 2 and 3). All the differences were considered significant at a level of $P < 0.05$.

Diagnosis by dermatologist

During the test, all subjects underwent a clinical diagnosis by one of the authors, a dermatologist, focusing on any adverse dermatological reactions such as inflammation, erythema or eczema.

Product characteristics

Procyanidin B-2 [epicatechin-(4β→8)-epicatechin] (213 g; purity, > 94%(w/w)) was obtained from twenty

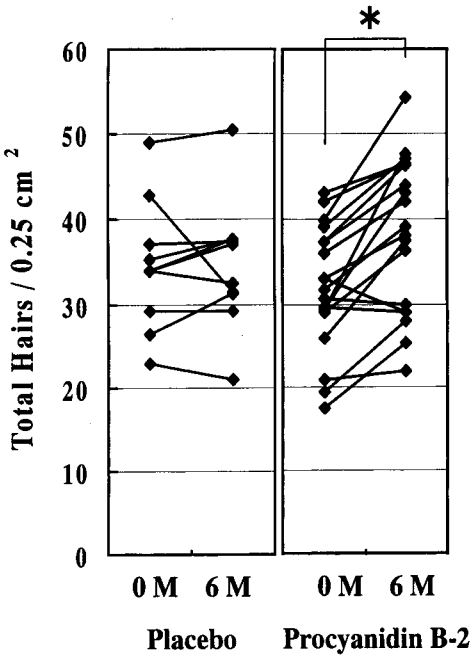

Fig. 2. Change in hair density. The number of total hairs in the designated area (0.5 cm square = 0.25 cm^2 area) after 6 months (6 M) of procyanidin B-2 treatment significantly increased over the baseline (0 M) figure for each subject ($^*P < 0.001$, paired t test); on the other hand, no significant difference was observed in the placebo controls (paired t test).

kiloliters of apple juice (*Malus pumila* Miller var. *domestica* Schneider, Fuji variety, commercial juice) according to the method described in a previous report (Takahashi et al., 1999a). The product was identified using mass spectrometry, ^{1}H-NMR and ^{13}C-NMR (Thompson et al., 1972; Morimoto et al., 1986).

Preparation of hair tonic for the clinical trial

One percent (w/w) of procyanidin B-2, 70% (w/w) of ethanol, 10% (w/w) of 1,3-butylene glycol, 0.5% (w/w) of *N*-acetylglutamine – isostearyl ester (Kyowa Hakko Kogyo Co., Japan), 0.25% (w/w) of polyoxyethylene (25) glyceryl monopyroglutamate monoisostearate (Nihon Emulsion Co., Japan), 0.1% (w/w) of *dl*-α-tocopherol, 0.05% (w/w) of *d*-biotin, 0.1% (w/w) of ascorbyl palmitate, 0.001% (w/w) of β-carotene, 0.1%(w/w) of sodium citrate, and 17.899% (w/w) of purified water were uniformly mixed to prepare the test sample. Vehicle without procyanidin B-2 was used as the placebo control. The placebo preparation was identical to the treatment preparation in smell, appearance, and consistency; ensuring that subjects would be unable to identify whether their test sample was the placebo or the treatment preparation. In addition, both preparations were put into the same black, opaque bottles.

Compliance

A useful measure for the subjects was the consumption of one 50-ml bottle within 2 weeks. The amount of agent habitually used was checked by the weighing the bottle after 2 weeks of usage. Additionally, the subjects were obliged to keep a diary on the use of the agent and any observations in connection with it.

Ethical Approval

Results of toxicological studies on procyanidin B-2 (Takahashi et al., 1999b) indicate that the safety of topical procyanidin B-2 has been thoroughly secured. Additionally, we performed an allergy test (challenge test) on 44 volunteers by means of 24-hour closed patches after a 2-week sequential application of 1%(w/w) procyanidin B-2 preparation following a 2-week period of no treatment. The results were all negative. The proposed human volunteer test passed Kyowa Hakko Kogyo Co., Ltd.'s Safety Commission. An Informed Consent contract was agreed between individual subjects and the company, confirming their willingness to participate in the test, their freedom to drop out at any time, confirming the acceptance by the company of responsibility for any accidents caused by procyanidin B-2 application, and safeguarding the confidentiality of individual information.

Results

Withdrawals

Data were available from 29 of the 30 patients who began the trial. One person in the procyanidin B-2 group was transferred to another location and thus dropped out. At the end of the test, 19 men in the procyanidin B-2 group and 10 men in the placebo group were subjected to analyses. No other persons dropped out for any reason.

Background factors

We confirmed that there were no significant differences between the procyanidin B-2 group and the placebo control group subjects with regard to distribution of background factors such as age or type and degree of baldness (Table 1).

Side effects

Dermatological diagnosis revealed no adverse side effects caused by these agents in either group: no inflammation, irritation or allergic reactions of the scalp were observed in any of the subjects; and no subjects complained of itchiness, pain, dryness or scaling of the scalp.

Changes in hair density

In the procyanidin B-2 group, the increase in number of total hairs in the designated area (0.5 cm square = 0.25 cm^2) after the 6-month trial was 6.68 ± 5.53 (mean ± SD)/0.25 cm^2, whereas in the placebo control group, the increase in number of total hairs was 0.08 ± 4.56 (mean ± SD)/0.25 cm^2 (Table 2). It is calculated that the increased number of total hairs in the designated area of procyanidin B-2 group subjects after the 6-month trial was significantly greater than that of the placebo control group subjects ($P < 0.005$, two-sample t test). The number of total hairs in the designated area (0.5 cm square = 0.25 cm^2) after 6 months of procyanidin B-2 treatment significantly increased over the baseline figure for each subject ($P < 0.001$, paired t test); on the other hand, no significant difference was observed in the placebo controls (paired t test)(Fig. 2).

Terminal hair formation

In the procyanidin B-2 group, the increase in number of terminal hairs (defined as hairs of > 60 μm in diameter) in the designated area (0.5 cm square = 0.25 cm^2) after the 6-month trial was 1.99 ± 2.58 (mean ± SD)/0.25 cm^2, whereas in the placebo control group, the increase in number of terminal hairs was −0.82 ± 3.40 (mean ± SD)/0.25 cm^2 (Table 2). It is calculated that the increased number of terminal hairs in the designated area of the procyanidin B-2 group subjects after the 6-month trial was significantly greater than that seen in the placebo control group subjects ($P < 0.02$, two-sample t test). The number of terminal hairs in the designated area (0.5 cm square = 0.25 cm^2) after 6 months of procyanidin B-2 treatment significantly increased over the baseline figure for each subject ($P < 0.005$, paired t test); on the other hand, no significant difference was observed in the placebo controls (paired t test)(Fig. 3).

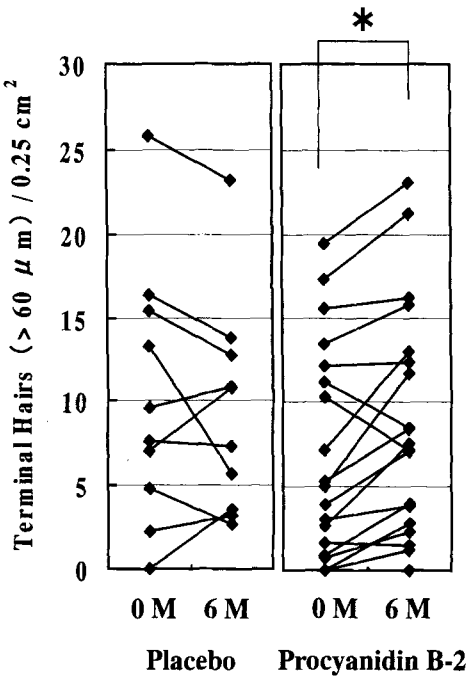

Fig. 3. Change in hair density. The number of terminal hairs in the designated area (0.5 cm square = 0.25 cm^2 area) after 6 months (6 M) of procyanidin B-2 treatment significantly increased over the baseline (0 M) figure for each subject (*P< 0.005, paired t test); on the other hand, no significant difference was observed in the placebo controls (paired t test).

Discussion

External application of procyanidins

Proanthocyanidins are a species of polyphenol known to possess a variety of physiological activities such as radical scavenging activity *in vitro* (Vennat et al., 1994), anti-oxidative properties *in vitro* (Hong et al., 1995), anti-mutagenic behavior *in vitro* (Liviero et al., 1994), anti-tumor-promoting behavior *in vivo* (Gali et al., 1994), anti-fungal effects *in vitro* (Eberhardt and Young, 1994), anti-viral behavior both *in vitro* and *in vivo* (Barnard et al., 1993), anti-dental-plaque formation *in vivo* (Matsudaira et al., 1998), anti-ulcer effects *in vivo* (Vennat et al., 1989), anti-allergic activity *in vitro* (Kanda et al., 1998), and anti-hypertensive activity *in vivo* (Cheng et al., 1993). Proanthocyanidins have been used as medications aimed at protecting the capillary vessels (Dartenuc et al., 1980); as preventive nutrition; and as cosmetics to protect the skin (Wayne, 1996). However, except for our previous reports (Takahashi et al., 1998; Takahashi et al., 1999a), there have been no reports on the hair-growing activity of proanthocyanidins. Proanthocyanidins in commercial use comprise a mixture of many proanthocyanidin molecules with different degrees of polymerization, which take the form of polymers or oligomers built of various flavan-3-ol units with a range of connection modes, and in some cases combine with other molecules such as gallic acid. Procyanidin B-2 can be obtained from the roots of *Fragaria vesca* (Vennat et al., 1988), from the leaves of *Melastoma candidum* (Cheng et al., 1993), or from the inner bark of *Pseudotsuga menziesii* (Douglas fir)(Gali et al., 1994), whereas we obtained highly purified procyanidin B-2 from apple juice (Takahashi et al., 1999a). In this report, we investigated the effects on the scalp and hair of highly purified procyanidin B-2 from apple juice. Our experimental results, based on close examination of 19 men taking part in a 6-month clinical trial, indicate that topical application of 1%(w/w) procyanidin B-2 of high purity is unlikely to show side effects such as irritation.

Efficacy on hair growth

Male pattern baldness is defined as a hair disease characterized by the miniaturization of terminal hairs (Sullivan and Kossard, 1998) and the vellus transformation of the hair (van Scott and Ekel, 1958). For the evaluation of hair-growing effects, three principal approaches have been reported; these are invasive (= biopsies) (Headington, 1984), semi-invasive (= epilations)(van Scott et al., 1957; Jackson et al., 1972; Rushton et al., 1983), and non-invasive methods. For non-invasive methods, the phototrichogram technique (Guarrera and Ciulla, 1986; Rushton et al., 1993; Van Neste et al., 1994), the unit area trichogram method (Rushton et al., 1990; Rushton et al., 1991) or the method based on analysis of the diameter of clipped hairs (Tsuji et al., 1994; Ishino et al., 1994) or in weight (Cottington et al., 1977; Price and Menefee, 1990) have been described. In this paper, we report on the approach of measuring changes in number of total and terminal hairs in the designated area by the macrophotography method in combination with the method of measuring hair diameter. The site analyzed before and after the 6-month period was not strictly the same; however, the difference in site determined using this method was confirmed to be within 0.5 cm. This is clearly an easy method; however, this method is also ideal for handling a large number of subjects since it has the advantages of speed and simplicity. In spite of the small number of subjects and the short period over which the trial was carried out, we observed a clear trend and significant results which were revealed by some statistical analyses towards increased hair density and terminal hair formation in the procyanidin group results (Figs. 2 and 3, Table 2). It is thought that application of procyanidin B-2 causes an increase in the anagen ratio, leading to increased hair density and terminal hair formation. The same effects have been reported with minoxidil (Savin, 1987; Roberts, 1987; Kreindler, 1987) and finasteride (an inhibitor of type II 5α-reductase, Merck)(Kaufman et al., 1998) therapy for androgenetic alopecia. In 2 % minoxidil treatment, an increase of 250 total hairs/5.1 cm^2 (calculated as 12.3 total hairs/0.25 cm^2) after 12-month therapy was reported (Kreindler, 1987). In finasteride treatment (1 mg/day, oral administration), an increase of 86 total hairs/5.1 cm^2 (calculated as 4.22 total hairs/0.25 cm^2) after 12-month therapy was reported (Kaufman et al., 1998). We observed an increase of 6.7 total hairs/0.25 cm^2 after 6 – months of procyanidin therapy (Table 2). The level of efficacy of 1 % procyanidin B-2 is concluded to compare favorably with minoxidil and finasteride therapy. It has also been reported that the anagen ratio undergoes seasonal changes: it rises to a maximum in March and falls to a minimum in September (Randall and Ebling, 1991). Our clinical test was performed from January to July, so the effects of seasonal changes are unlikely to have influenced the overall results.

Assumed mechanism of action of procyanidin B-2

The main mechanism of action of procyanidin B-2 is implied by its intensive growth-promoting action on hair epithelial cells (Takahashi et al., 1999a). The intensive anti-oxidative activity of procyanidin B-2 may be a significant contributor to its effects. The relation between male pattern baldness and inflammation has been pointed out by several researchers. It has been reported that lymphocytic inflammation was observed around hair follicles biopsied from patients showing male pattern baldness (Sueki et al., 1999; Jaworsky et al., 1992). Young et al. (1991) also reported that the ratio of subjects showing inflammation of the scalp was 100 % in subjects with male pattern baldness, whereas the ratio was 66 % in non-balding subjects. Procyanidins are known to show the effect of decreasing inflammation due to their anti-oxidative properties (Haslam, 1996) and by their protease inhibiting action (Tixier et al., 1984). Therefore, it is supposed that the suppression of inflammation mediated by procyanidin B-2 returns the scalp to a healthy condition, consequently leading to a cure for baldness. The hair-growing activity of procyanidin B-2 may depend on more than one of the numerous physiological functions of this compound.

Conclusions

These results show that the agent comprising procyanidin B-2 exhibits excellent hair-growing effects. The increased number of total and terminal hairs of the subjects in the procyanidin B-2 group were significantly greater than those in the placebo controls (Figs. 2 and 3, Table 2). No deleterious effects on the scalp were seen. We have confirmed from our tests that topical procyanidin B-2 is safe and effective for curing male pattern baldness and are encouraged to proceed to large scale clinical trials. We are now planning dose-ranging and period of time experiments. Also, we have improved the industrial process for manufacturing procyanidin oligomers from immature apples (Yanagida et al., 2000).

Acknowledgments

I am grateful to Dr. H.-F. Leu for her support and to Mr. R. Katakura and Ms. Y. Ohishi for their technical assistance.

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