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Promotion effect of norgalanthamine, a component of *Crinum asiaticum*, on hair growth

This study was conducted to evaluate the effect of *Crinum asiaticum*, a plant native to Jeju Island, Korea, on the promotion of hair growth. When rat vibrissa follicles were treated with a 95% ethanol (EtOH) extract of *C. asiaticum*, the hair-fiber lengths of the vibrissa follicles increased significantly. In addition, after daily topical application of the EtOH extract of *C. asiaticum* onto the back of C57BL/6 mice, anagen progression of the hair shaft was induced. Moreover, the extract increased the proliferation of immortalized vibrissa dermal papilla cells. When the vibrissa follicles in the anagen phase were treated with the extract, immunohistochemical analysis revealed that the extract was found to increase the expression of proliferating cell nuclear antigen (PCNA) in the bulb region of the 7-day cultured follicles. In particular, norgalanthamine, a principal of the extract, showed activity that increased the hair-fiber lengths of vibrissa follicles and the proliferation of dermal papilla cells. These results suggest that norgalanthamine, a principal of *C. asiaticum*, has the potential to promote hair growth via the proliferation of dermal papilla.

Key words: *Crinum asiaticum*, hair growth, norgalanthamine, vibrissa follicle, C57BL/6 mice, dermal papilla cell

The hair follicle is a small and dynamic organ which periodically synthesizes the biological fibers termed as hair [1]. The cyclic change of the hair follicle, which occurs over the entire lifetime of a mammal, involves a growth phase (anagen), an involution phase (catagen) and a resting phase (telogen) [2].

Recently, there has been an increasing number of people suffering from hair loss or thinning [3, 4]. Androgenetic alopecia (AGA), which is the most common type of alopecia, is a common problem in men over the age of 40. However, the underlying causes of baldness are poorly understood [3, 5]. Many materials have been used to cure alopecia. However, only two drugs so far have been approved for hair loss treatment by the Food and Drug Administration (FDA, USA); *i.e.*, finasteride and minoxidil [3, 6]. Finasteride is a type II 5α-reductase inhibitor. It was initially used for curing prostatic hypertrophy [7] but later found to stimulate hair growth [8-10]. Nevertheless, its use is limited because of potential side effects, especially in women [11]. Minoxidil was originally synthesized as a potassium channel opener and was further developed as an anti-hypertensive. Moreover, it was also found to stimulate the growth of hair follicle cells *in vitro* [12] and to have hair cycle converting activity *in vivo* [13]. Recently, Han *et al.* [14] reported that minoxidil has proliferative and anti-apoptotic effects on dermal papilla cells. The dermal papilla cells consist of a cluster of specialized fibroblasts that play important roles in the regulation of the hair follicle through the secretion of diffusible proteins. Such proteins include insulin-like growth factor-1(IGF-1) [15], hepatocyte growth factor/scatter factor (HGF/SF) [16] and fibroblast growth factor-7 (FGF-7) [17]. In addition, other growth factors are also found to be involved in hair growth regulation. For example, vascular endothelial growth factor (VEGF) [18] and keratinocyte growth factor (KGF) [19] have a stimulatory effect on hair follicle growth, while transforming growth factor-β (TGF-β) [20], fibroblast growth factor-5 (FGF-5) [21], epidermal growth factor (EGF) [22], interleukin-1α (IL-1α) [23], and interleukin-1β(IL-1β) [24] are known to negatively regulate hair growth.

To develop new therapies to enhance hair growth, we screened the extracts of plants that have traditionally been used in oriental medicine and discovered that *Crinum asiaticum* has the best promoting effect. *Crinum asiaticum* var. *japonicum* (Amaryllidaceae) is only distributed in Korea and Japan. In Korea, the plant has been used as a rheumatic remedy, an anti-pyretic, an anti-ulcer treatment, and for the alleviation of local pain and fever. Regarding phytochemical studies on this plant, the isolation of phenanthridine alkaloids, sterols, flavonoids and triterpene alcohols have been previously reported [25, 26]. Alkaloids isolated from the bulbs of Amaryllidaceae plants have shown various pharmacological and microbiological effects, such as antiviral [27], antimalarial [28], cytotoxic [28-30], and antineoplastic activities [31], as well as effects on diseases of the nervous system [32]. In a report by the same authors, *C. asiaticum* was also reported to have an anti-inflammatory effect by inhibition

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of iNOS and release of PGE2, IL-6, and IL-8, which are known as the cytokines associated with inflammation [33]. In addition, crinamine from *C. asiaticum* has been reported to inhibit HIF-1 activity [34]. Alkaloids with the galanthamine-type skeleton, isolated from the Amaryllidaceae family, are potent acetylcholinesterase (AChE) inhibitors [35] and have been used to treat the symptoms of Alzheimer's disease [36]. It has been reported that lycorine has antitumor activity [29]. However, the promotion effect of *C. asiaticum* on hair growth has not yet been reported.

Therefore, the present study was carried out to investigate the promotion effect of the extract of *C. asiaticum*, as well as norgalanthamine and lycorine (isolated alkaloids from *C. asiaticum)*, on the growth of hair.

Materials and methods

Materials

The 95% EtOH extract of *Crinum asiaticum* and lycorine were kindly provided by R&D center, Bioland Ltd (Chungnam, Korea). Norgalathamine was generously gifted by Dr Young Ho Kim (Chungnam National University, Chungnam, Korea). Their chemical structures are shown in *figure 1*, and were freshly dissolved in dimethyl sulfoxide (DMSO) (Sigma, Mo, USA) for subsequent treatment.

Animals

Male Wistar rats (3 weeks of age) were supplied from Orient Bio (Seongnam, Gyeonggi, Korea). 6-week-old female C57BL/6 mice were purchased from Dae-Han Biolink (Eumsung, Chungbuk, Korea) and provided with a standard laboratory diet and water *ad libitum*. All animals were cared for by using protocols (20070002) approved by the Institutional Animal Care and Use Committee (IACUC) of the Jeju National University.

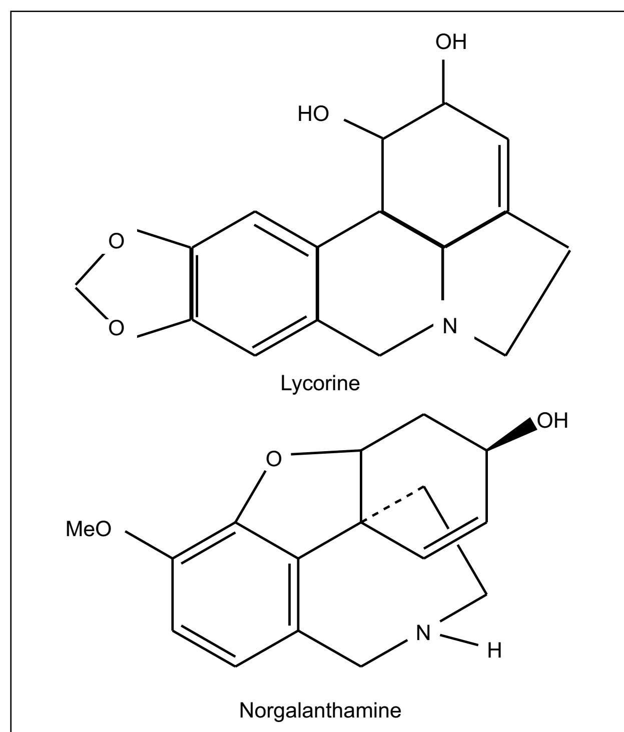

Figure 1. Structures of lycorine and norgalanthamine.

Cell Culture

Rat vibrissa immortalized dermal papilla cell line [37] was donated by the Skin Research Institute, Amore Pacific Corporation R&D Center, South Korea. The dermal papilla cells were cultured in DMEM (Hyclone Inc, UT, USA) supplemented with 10% fetal bovine serum (Gibco BRL, NY, USA) and penicillin/streptomycin (100 unit/mL and 100 µg/mL, respectively) at 37 °C in a humidified atmosphere under 5% CO_2.

Isolation and culture of rat vibrissa follicles

Isolation of rat vibrissa follicles was performed as described previously [38]. Briefly, rat vibrissa follicles were harvested from male Wistar rats that were 23 days old. To accomplish this, the rats were sacrificed under diethyl ether. Next, both the left and right mystacial pads were removed from the rats and placed in a 1:1 (vol/vol) solution of Earle's balanced salts solution (EBSS, Sigma, MO, USA) and phosphate buffered saline (PBS, Sigma, MO, USA) that contained 100 unit/mL of penicillin and 100 µg/mL of streptomycin. Anagen vibrissa follicles were then carefully dissected under a stereomicroscope (Olympus, Tokyo, Japan) from posterior parts of the mystacial pads with considerable care being taken to remove the surrounding connective tissue without damaging the vibrissa follicle. Using this method we were able to routinely isolate more than 40 follicles from each animal. The isolated follicles were then placed in separate wells in 24-well plates that contained 500 µL of Williams medium E (Gibco Inc, NY, USA) supplemented with 2 mM L-glutamine (Gibco Inc, NY, USA), 10 µg/mL insulin (Sigma, MO, USA), 50 nM hydrocortisone (Sigma, MO, USA), 100 unit/mL penicillin and 100 µg/mL streptomycin at 37 °C and cultivated in an atmosphere comprised of 5% CO_2 and 95% air. The isolated follicles were then treated with vehicle (DMSO diluted 1:1000 in Williams medium E) as a control, *C. asiaticum* extract (0.01 ~ 100 µg/mL), lycorine (0.001 ~ 0.1 µM) and norgalathamine (0.001 ~ 0.1 µM). Minoxidil sulfate (MS) (Sigma, MO, USA) was used as a positive control in the culture systems [39]. The culture medium was changed every 3 days and photographs of the cultured rat vibrissa follicles were taken using a stereomicroscope for 3 weeks. The length of the hair follicles was measured using a DP controller (Olympus, Tokyo, Japan).

Hair growth activity *in vivo*

Anagen was induced by depilation on the back skin of C57BL/6 mice that were in the telogen phase of the cycle, as described previously [40]. Briefly, 6 week old female C57BL/6 mice were allowed to adapt to their new environment for one week. The anagen was then induced in the back skin of the seven week old female C57BL/6 mice by shaving, which led to synchronized development of anagen hair follicles. From the following day (day 1), 0.2 mL of a 1 mg/mL solution of *C. asiaticum* extract in 50% ethanol was topically applied every day for 31 days. 5% Minoxidil (MINOXYL^{TM}; Hyundai Pharm. Co. Ltd., Cheonan, Chungnam, Korea) was used as a positive con-

trol. The back skin of the mice was then observed and photographed at 1, 10, 17 and 31 days after shaving.

MTT Assay

The proliferation of dermal papilla cells was evaluated by measuring the metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [41]. Briefly, dermal papilla at 1.0×10^4 cells/mL were seeded into 96-well plate, cultured 24 h in serum-free DMEM, and then treated with vehicle (DMSO diluted 1:1000 in serum-free DMEM) as a control, *C. asiaticum* extract (0.01 ~ 10 μg/mL), lycorin (0.001 ~ 0.1 μM) and norgalathamine (0.001 ~ 0.1 μM) for 4 days. After incubation, 0.1 mg (50 μL of a 2 mg/mL solution) of MTT (Sigma, MO, USA) was added to each well, and the cells were then incubated at 37 °C for 4 h. Next, the plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. 200 μL of dimethyl sulfoxide was then added to each well to dissolve the formazan crystals and the absorbance of the plates at 540 nm was then read immediately on a microplate reader (BioTek Instrument, Inc., VT, USA). All experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the percentage reduction in the absorbance caused by treatment with the extract or the active component compared to that of the untreated controls.

Immunohistochemistry

For immunohistochemistry, vibrissa follicles were collected from each group on days 0 and 7 of treatment. The vibrissa follicles were fixed in 4% paraformaldehyde (Sigma-Aldrich, MO, USA), and the tissues were then dehydrated and embedded in paraffin. Immunohistochemistry was then performed according to the manufacturer's instructions (Santa Cruz Biotechnology, CA, USA). The following primary antibodies were used at the indicated concentrations: PCNA (1:200; Santa Cruz Biotechnology, CA, USA). In addition, the relevant goat secondary antibodies (1:200; Santa Cruz Biotechnology, CA, USA) were used for detection of the primary antibodies.

Statistical analyses

The hair growth data are expressed as the mean of the follicle lengths ± the standard errors (SE) of at least three independent experiments performed in triplicate. The Student's t-test was used to determine the statistical significance (P-value < 0.05) of the differences between the values for the various experimental and control groups.

Results

The effects of *C. asiaticum* extract on rat vibrissa follicle elongation

To determine if *C. asiaticum* induced hair growth, we first examined the activity of *C. asiaticum* extract using an organ culture of the rat vibrissa follicle. When rat vibrissa follicles were treated with various concentrations of *C. asiaticum* extract for 3 weeks, the hair-fiber length of the vibrissa follicles was significantly increased in a dose-dependent manner with respect to the control *(figure 2)*. In particular, in the vibrissa follicle that was treated with 10 μg/mL of *C. asiaticum* extract for 21 days, the vibrissa follicles were 177.3 ± 30.7% longer ($P < 0.05$) than those in the control group. These results indicate that *C. asiaticum* extract is capable of promoting hair growth.

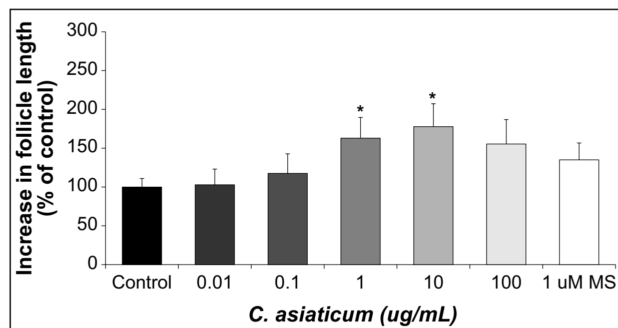

Figure 2. Hair growth effects of 95% EtOH extracts from *C. asiaticum* on rat vibrissa follicles. Individual vibrissa follicles from Wistar rats were microdissected and then cultured in William's E medium at 37 °C under 5% CO_2. Vibrissa follicles were then treated with various concentrations of 95% EtOH extract from *C. asiaticum* for 21 days. Stimulation with minoxidil sulfate (MS) served as a positive control. All experiments were performed in triplicate. The difference in the length of vibrissa follicles of the control group on day 21 was taken to be 100%. Data are presented as the percentage of the length of the treated follicles based on the mean length of the control follicles ± the SE. Vibrissa follicles treated with *C. asiaticum* showed a significant increase in hair-fiber length when compared with the control. Moreover, the hair-fiber length of vibrissa follicles that were treated with *C. asiaticum* was greater than those of the positive control. *$P < 0.05$ *vs*. Control.

The effects of *C. asiaticum* extract on anagen induction in C57BL/6 mice

To investigate whether anagen induction was promoted by *C. asiaticum*, we used C57BL/6 mice. C57BL/6 mouse dorsal hair is known to have a time-synchronized hair growth cycle [40]. Shaved skin of telogen mice is pink and darkens along with anagen initiation. After being shaved, the skin color of the mice was observed to be pink. As shown in *figure 3, C. asiaticum* extract and minoxidil almost exhibited gray skin at 10 days post-hair growth induction, and their hair shafts were visible at 14 days (data not shown). The control groups remained pink until day 10 and exhibited gray skin by day 12 (data not shown). The hair shafts of the control groups were first visible on days 15 (data not shown) to 17. On the 31st day, the back skin was in the anagen phase in all the mice. Overall, these results indicate that *C. asiaticum* extract induced early telogen-to-anagen conversion of hair follicles in the C57BL/6 mice.

The effects of *C. asiaticum* extract on cell proliferation of hair follicles

To evaluate the effect of *C. asiaticum* on cell proliferation of hair follicles, proliferation of dermal papilla cells and the expression of PCNA were examined.

Immortalized rat vibrissa dermal papilla cells were treated with various concentrations of *C. asiaticum* extract, and the mitogenic effect on the dermal papilla cells was exam-

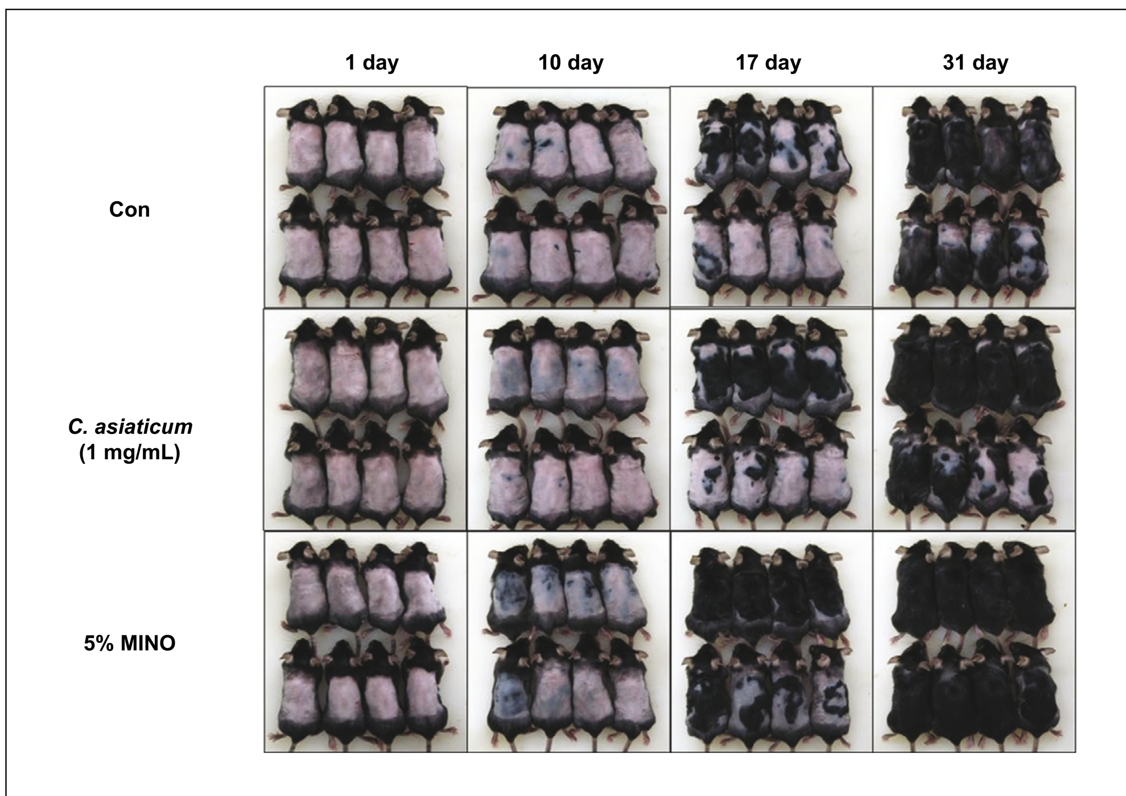

Figure 3. The effect of 95% EtOH extracts from *C. asiaticum* on anagen induction in C57BL/6 mice. After shaving, the back skins were treated with *C. asiaticum* extract every day for 31 days. The back skins were photographed at 1, 10, 17 and 31 days after depilation. Skin darkness was increased in *C. asiaticum* treated mice, whereas that of control mice was less affected.

ined. *C. asiaticum* extract promoted the proliferation of dermal papilla cells at a concentration of 0.1 μg/mL compared with the vehicle-treated control. However, 0.01, 1 and 10 μg/mL of *C. asiaticum* extract did not affect the proliferation of dermal papilla cells *(figure 4)*. These results suggest that the hair growth promoting effect of *C. asiaticum* extract may be mediated through a mitogenic effect on dermal papilla cells. The isolated rat vibrissa follicles were treated with *C. asiaticum* extract and then examined for activation of PCNA *(figure 5)*. In the anagen vibrissa follicles (0 day), the expression of PCNA was positively stained in the bulb region, whereas the 7-day cultured vibrissa follicles, which were expected to be in the anagen-catagen transition phase, were negatively stained. The vibrissa follicles treated with 10 μg/mL *C. asiaticum* extract for 7 days were positively stained in the bulb regions. In addition, the bulb regions of the vibrissa follicles that were treated with 1 μM minoxidil sulfate for 7 days were positive for PCNA. These results indicate that the cells in the bulb regions of follicles treated with *C. asiaticum* extract or minoxidil sulfate were induced to grow *(figure 5)*.

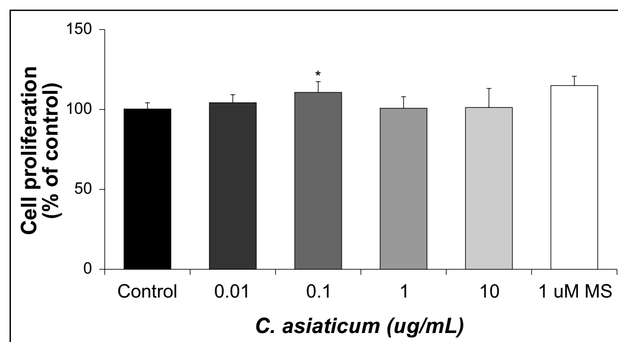

Figure 4. Proliferation effect of 95% EtOH extracts from *C. asiaticum* on cultured dermal papilla cells. Rat dermal papilla cells (1.0×10^4 cells/mL) were plated in 96 well plates. Dermal papilla cells were treated with various concentration of *C. asiaticum* extract or minoxidil sulfate (MS), as indicated. Cell proliferation was measured using a MTT assay for 5 days. All experiments were performed in triplicate. Data are presented as the mean ± the SD. $*P < 0.05$ *vs*. control.

The effects of isolated compounds from *C. asiaticum* on the promotion of hair-growth

We next examined which compounds are responsible for the hair growth promoting activity of *C. asiaticum*. As shown in *figure 6A*, when rat vibrissa follicles were treated with lycorine (the main component of *C. asiaticum)* at 0.001, 0.01 and 0.1 μM for 21 days, a hair growth effect was not shown. On the other hand, norgalanthamine, another component of *C. asiaticum*, significantly increased hair growth promoting activity by 110.3 ± 14.4% at a dose of 0.001 μM, 128.7 ± 13.8% at a dose of 0.01 μM and 139.2 ± 10.3% at a dose of 0.1 μM compared, with the control group. As shown in *figure 6B*,

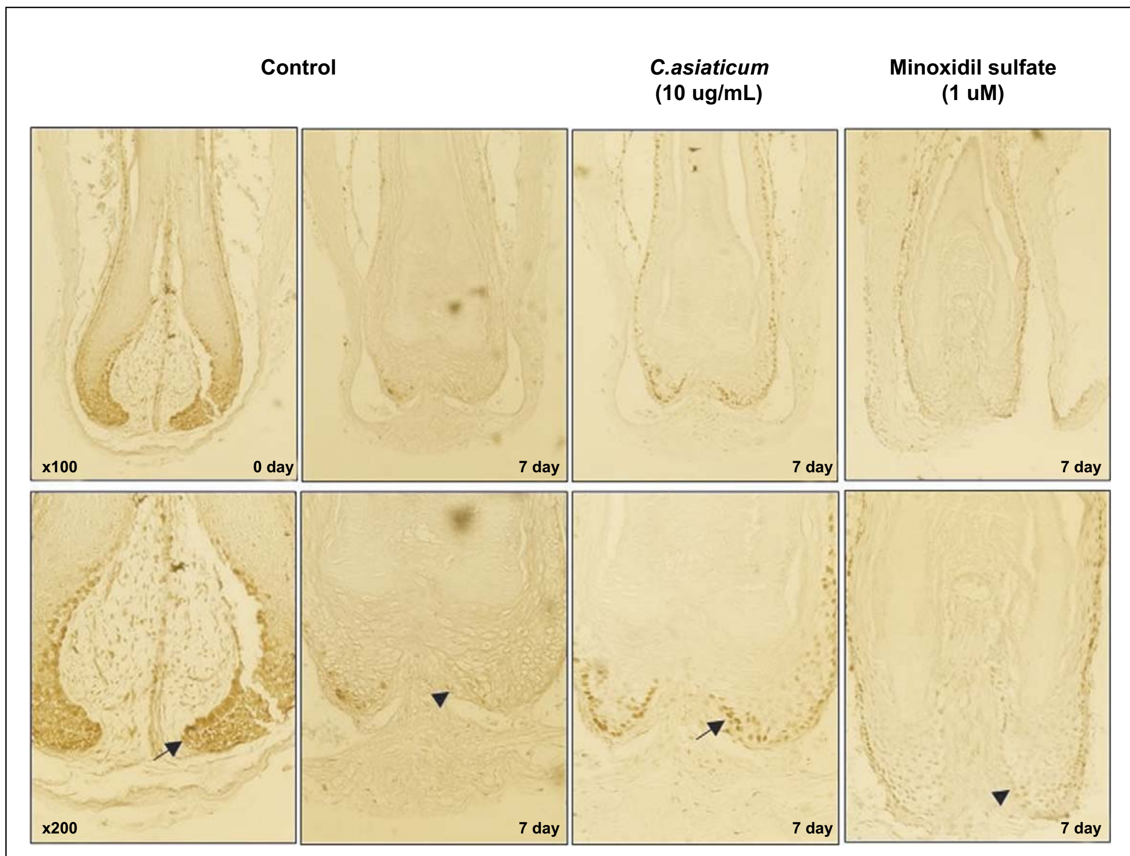

Figure 5. The effect of 95% EtOH extracts from *C. asiaticum* on PCNA expression in vibrissa follicles. Vibrissa follicles were treated with *C. asiaticum* extract or minoxidil sulfate for 7 days. Sections of the vibrissa follicles were stained with anti-PCNA antibody. In anagen vibrissa follicles (0 day), the expression of PCNA was positively stained in the bulb region (indicated by *arrows*). The 7-day cultured vibrissa follicles that were expected to be in the anagen-catagen transition phase were negatively stained (indicated by *arrowheads*), whereas the vibrissa follicles treated with 10 μg/mL of the extract of *C. asiaticum* for 7 days were positively stained in the bulb region (indicated by *arrow*). In addition, the bulb regions of the vibrissa follicles treated with 1 μM of minoxidil sulfate for 7 days were positive for PCNA (indicated by *arrowheads*).

when immortalized rat vibrissa dermal papilla cells were incubated with 0.01 μM norgalanthamine for 4 days, the immortalized rat vibrissa dermal papilla cells were 114.0 ± 4.3% longer ($P < 0.05$) than those in the control group. However, lycorine did not affect the proliferation of dermal papilla cells. Therefore, norgalathamine is very likely responsible for the hair growth promoting activity of *C. asiaticum*.

Discussion

In this study, the hair growth promoting effects of *C. asiaticum in vitro* and *in vivo* were investigated. To the best of our knowledge, this study is the first to demonstrate that *C. asiaticum* and norgalanthamine, a main principal of *C. asiaticum*, have the potential to promote hair growth via the proliferation of dermal papilla.

The difficulties in developing effective therapies for hair growth lie in the fact that a single proper evaluation method has not yet been established. In 1990, Philpott *et al.* demonstrated that a human hair follicle could be cultured organo-typically *in vitro* [42]. Using a similar experimental technique, culture of the hair follicle from many other species, such as rat, sheep and horse, has also been successfully established [43, 44]. Many investigators have adopted hair follicle culture models to evaluate the effects of several compounds [45, 46]. Moreover, vibrissa follicles from rats are much larger than pelage follicles and can be successfully cultured *in vitro* [39]. In particular, the hair growth cycles of the rat vibrissa follicles have been reported to be synchronized according to their age [47] and large posterior vibrissae of 21 d rats have been shown to be always in anagen and show no sign of catagen [38]. The isolated rat vibrissa follicles could be maintained *in vitro* up to 23 days and then the follicles may enter into catagen phase [38]. Recently, an *in vitro* culture system for murine vibrissae to reinitiate anagen was developed [48]. In this study we isolated large posterior vibrissa follicles from 23 d old rats and maintained them for up to 21 d *in vitro*.

In the continuing search for hair-growth promoting compounds from natural sources, we have examined more than 5 kinds of natural products for growth promoting effects with rat vibrissa *in vitro* culture system. We found that the extract of *Schisandra nigra* [49] and *C. asiaticum* extract increased hair-fiber length in cultured rat vibrissa follicles, whereas *Schisandra chinensis, Cudrania tricuspidata* and *Eclipta prostrate* extract did

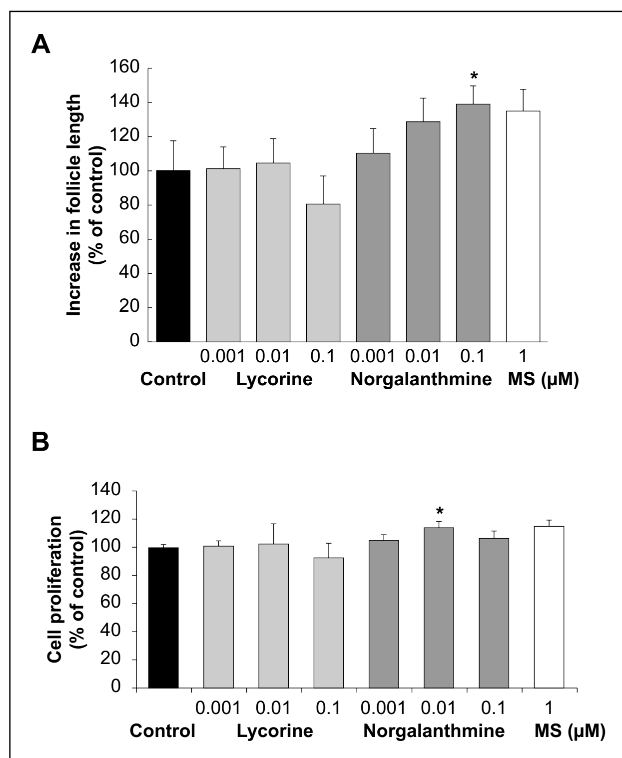

Figure 6. The effects of isolated compounds from *C. asiaticum* on the promotion of hair-growth. **A)** Hair growth effects of isolated compounds from *C. asiaticum* on rat vibrissa follicles. Vibrissa follicles were treated with 0.001, 0.01 and 0.1 μM of norgalanthamine or lycorine from *C. asiaticum* for 21 days Stimulation with minoxidil sulfate (MS) served as a positive control. All experiments were performed in triplicate. The difference in the length of vibrissa follicles of the control group on day 21 was taken to be 100%. Data are presented as the percentage of the length of the treated follicles based on the mean length of the control follicles ± the SE. $^*P < 0.05$ *vs.* Control. **B)** Proliferation effect of isolated compounds from *C. asiaticum* on cultured dermal papilla cells. Rat dermal papilla cells (1.0×10^4 cells/mL) were plated in 96 well plates. Dermal papilla cells were treated with 0.001, 0.01 and 0.1 μM of norgalanthamine or lycorine from *C. asiaticum* or minoxidil sulfate (MS), as indicated. Cell proliferation was measured using a MTT assay for 5 days. All experiments were performed in triplicate. Data are presented as the mean ± the SD. $^*P < 0.05$ *vs.* control.

not show hair growth-promoting activity (data not shown). Specifically, 10 μg/mL of *C. asiaticum* extract was found to induce a greater increase in hair-fiber length than minoxidil sulfate, a positive control. The use of organ culture methods to evaluate hair follicle growth is thought to be correlated with *in vivo* systems because the extent of hair growth can be observed as the sum of the function of each cell [42]. The hair growth promoting *in vitro* effect of *C. asiaticum* extract was also observed *in vivo* using C57BL/6 mice. The topical application of 5% minoxidil (MINOXYL^{TM}) promoted hair growth faster than *C. asiaticum*. This suggests at least the following possibilities: 1) The active component of *C. asiaticum* extract was absorbed into skin much less than minoxidil; 2) After being absorbed into the skin, the active component of *C. asiaticum* extract was metabolized to inactive metabolites faster than the minoxidil; 3) Another unexpected factor was involved in the *in vivo* activity.

The mesenchyme-derived dermal papilla cells play a pivotal role in hair growth regulation. The morphology of dermal papilla cells can be altered through the hair growth cycle, being maximal in volume in the growing phase (anagen) and least in the resting phase (telogen). Evidence has shown that the size of dermal papilla cells is well correlated with hair growth, and the cell number of dermal papilla cells is increased in the growing phase of hair cycle [50, 51]. Therefore, to investigate the effect of *C. asiaticum* on cell growth in the hair follicles, we examined whether *C. asiaticum* influenced the proliferation of dermal papilla cells. As shown in *figure 4*, *C. asiaticum* extract was found to increase the growth of dermal papilla cells. We further tested the expression of PCNA as an index of cell proliferation [52]. As shown in *figure 5*, immunohistochemical analysis revealed that *C. asiaticum* extract was found to increase the expression of PCNA in the bulb region of the 7-day cultured follicles. Taken together, the results of this study indicated that hair growth induced by *C. asiaticum* may be mediated through mitogenic effects that occur in the dermal papilla region.

C. asiaticum have been reported to contain various phenanthridine alkaloids, flavonoids and sterols [25, 26]. We have recently isolated crinamine, lycorine and norgalanthamine from *C. asiaticum* var. *japonicum* [33, 34]. Crinamine was reported to have strong cytotoxic activity and an HIF-1 inhibitory effect [34]. Lycorine also showed cytotoxic and anti-inflammatory activities [29, 33]. Galanthamine-type skeleton has been reported to have acetylcholinesterase (AChE) inhibition [35]. Therefore, we examined which compounds are responsible for the hair growth promoting activity of *C. asiaticum*. As shown in *figure 6*, lycorine, the main component of *C. asiaticum*, did not show hair growth, thereby indicating that it has cytotoxic activity. On the other hand, norgalanthamine, another component of *C. asiaticum*, showed activity that increased hair-fiber lengths of vibrissa follicles and proliferation of dermal papilla cells. Therefore, among the compounds tested, norgalanthamine is very likely responsible for the hair growth promoting activity of *C. asiaticum*.

Overall, the results of this study demonstrated that *C. asiaticum* is capable of promoting hair growth *in vitro* and *in vivo* via the proliferation of dermal papilla cells. In further studies, the mechanisms by which *C. asiaticum* and norgalanthamine promote hair-growth as well as other hair-growth promoting compounds should be elucidated. ■

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