INTRODUCTION

Hair plays an important role in biological functions, from providing protection to maintaining body temperature (Kim and Dao, 2020). Hair loss is a common disorder associated with hair thinning and loss of hair from the head, caused by a variety of factors, including stress, inflammation, hormonal imbalance, nutritional imbalance, diseases, and medication (Springer et al., 2003). Although hair loss is not a fatal disease, it is known to have detrimental effects on the psychological state of affected individuals, and its incidence rate is on the rise (Talavera-Adame et al., 2017).

Hair is generated in hair follicles over the three stages of the hair growth cycle to determine the growth, maintenance, and elimination of hair: (i) anagen phase (the active growth state of hair follicles); (ii) catagen phase (the regression state of hair follicles); (iii) telogen phase (the rest state of the hair follicles). Hair loss is caused by disorders in the hair growth cycle, such as the shortening of the anagen phase, the rapid entry of the catagen phase, and the prolongation of the telogen phase (Pantelireis and Higgins, 2018). Among the cells of the hair follicle, dermal papilla cells (DPCs), a mesenchymal-

Abstract

Alopecia is a distressing condition caused by the dysregulation of anagen, catagen, and telogen in the hair cycle. Dermal papilla cells (DPCs) regulate the hair cycle and play important roles in hair growth and regeneration. Myristoleic acid (MA) increases Wnt reporter activity in DPCs. However, the action mechanisms of MA on the stimulation of anagen signaling in DPCs is not known. In this study, we evaluated the effects of MA on anagen-activating signaling pathways in DPCs. MA significantly increased DPC proliferation and stimulated the G2/M phase, accompanied by increasing cyclin A, Cdc2, and cyclin B1. To elucidate the mechanism by which MA promotes DPC proliferation, we evaluated the effect of MA on autophagy and intracellular pathways. MA induced autophagosome formation by decreasing the levels of the phospho-mammalian target of rapamycin (phospho-mTOR) and increasing autophagy-related 7 (Atg7) and microtubule-associated protein 1A/1B-light chain 3II (LC3II). MA also increased the phosphorylation levels of Wnt/β-catenin proteins, such as GSK3β (Ser9) and β-catenin (Ser552 and Ser675). Treatment with XAV939, an inhibitor of the Wnt/β-catenin pathway, attenuated the MA-induced increase in β-catenin nuclear translocation. Moreover, XAV939 reduced MA-induced effects on cell cycle progression, autophagy, and DPC proliferation. On the other hand, MA increased the levels of phospho (Thr202/Tyr204)-extracellular signal regulated kinases (ERK). MA-induced ERK phosphorylation led to changes in the expression levels of Cdc2, Atg7 and LC3II, as well as DPC proliferation. Our results suggest that MA promotes anagen signaling via autophagy and cell cycle progression by activating the Wnt/β-catenin and ERK pathways in DPCs.

Key Words: Myristoleic acid, Dermal papilla cells, Anagen, Autophagy, Wnt/β-catenin, ERK

Myristoleic Acid Promotes Anagen Signaling by Autophagy through Activating Wnt/β-Catenin and ERK Pathways in Dermal Papilla Cells

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derived fibroblast located at the base of the hair follicle, play an important role in hair growth and in the regeneration of hair follicles (Greco et al., 2009). DPCs stimulate the initiation of anagen by generating instructive signals that induce epithelial bulge cell proliferation or by inducing stem cells (Chi et al., 2013). In addition, DPC proliferation occurs at the anagen phase, which is essential for maintaining hair follicles (Chi et al., 2013). At present, the US Food and Drug Administration (FDA) has only approved two drugs for the treatment of hair loss: minoxidil (MXD) and finasteride. Although the action mechanism of MXD remains unclear, it has been found to induce hair growth by inducing angiogenesis (increased gene expression of vascular endothelial growth factor (VEGF)), vasodilation (increased nutrient supply), and the opening of ATP-sensitive K⁺ channels (KᵥATP channels) (Meisher et al., 1988; Buhl et al., 1992; Lachgar et al., 1998). MXD has also been reported to extend the anagen phase by activating the Wnt/β-catenin pathway and improving hair loss by inhibiting apoptosis in DPCs (Han et al., 2004; Kwack et al., 2011). Finasteride has been demonstrated to prevent androgenic alopecia (also called male pattern hair loss) by inhibiting the activity of 5α-reductase type II, which affects male hormone metabolism (Mysore and Shashikumar, 2016). However, many side effects of MXD (relapse at discontinuation, erythema and itching) and finasteride (male sexual dysfunction, female infertility and birth defects) have been reported. Thus, these drugs should be used with caution (Melcangi et al., 2013). As a result, interest in supplementary and alternative treatments using safe reagents derived from plant has increased in recent years. Therefore, further research is needed on the development of therapeutic agents using natural products (Hosking et al., 2019).

Myristoleic acid (MA), also known as 9-tetradecenoic or myristoleate, is an omega-5 monounsaturated fatty acid obtained from the seeds of plant from the Myristicaceae family. It is biosynthesized in organisms from myristic acid by the enzyme stearylo-CoA desaturase (SCD)-1, also known as delta-9 desaturation. MA is present in all eukaryotic organisms and is found in human adipose tissue (Jiang et al., 1999). In addition, food products, such as milk, butter, and margarine are also known to contain MA (Couverre et al., 2006). It has been reported that MA has several effects, including osteoclast inhibition, anti-obesity, and anti-cancer effects on prostate cancer (Iguchi et al., 2001; Kwon et al., 2015; Quan et al., 2020). MA was reported to enhance Wnt reporter activity in DPCs (Lee et al., 2016). However, the intracellular mechanisms that underlie the effect of MA on the stimulation of anagen signaling in DPCs have not been investigated. In this study, we addressed the actions of MA to provoke anagen-activating signaling pathways in DPCs. Here, we report that MA can induce anagen signaling by autophagy and G2/M phase cell cycle progression through the activation of the Wnt/β-catenin and extracellular signal regulated kinase (ERK) pathways in DPCs.

MATERIALS AND METHODS

Reagents
MA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Fig. 1A). DMSO, bovine serum albumin (BSA), and MXD were purchased from Sigma-Aldrich. Fetal bovine serum (FBS), penicillin-streptomycin solution and trypsin-EDTA solution were obtained from Gibco (Grand Island, NY, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Dulbecco’s phosphate-buffered saline (DPBS) was obtained from WelGENE (Daegu, Korea). EZ-CYTOX, a water-soluble tetrazolium (WST)-based cell viability assay kit, was purchased from Daile Lab Service (Seoul, Korea). PRO-PREP protein extraction solution was obtained from Invitrogen Biotechnology (Seoul, Korea). Polyvinylidene fluoride (PVDF) membranes were purchased from Bio-Rad (Hercules, CA, USA). Westar Nova 2.0 ECL solution was obtained from Cyanagen (Bologna, Italy). X-ray film was purchased from Agfa-Gevaert (Mortsel, Belgium). 4% paraformaldehyde (PFA) solution was purchased from Bioesang (Seongnam, Korea). The reagents used for western blotting and immunofluorescence staining included anti-cyclin A, -Cdc2, -cyclin B1, -β-actin, -β-catenin, -α-tubulin, horseradish peroxidase (HRP)-labeled anti-rabbit IgG, and HRP-labeled anti-mouse IgG, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho (Ser2448)-mTOR, -Atg7, -LC3II, -GSK3β, -phospho (Ser473)-β-catenin, -phospho (Ser9)-β-catenin, -ERK, and -phospho (Thr8/Tyr18) ERK were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-LC3II was purchased from Abcam (Cambridge, MA, USA). Anti-mouse AlexaFluor® 488, -rabbit AlexaFluor® 594, and -rabbit AlexaFluor® 488 were purchased from Invitrogen (Carlsbad, CA, USA). 2-phenylindole (DAPI)-contained VECTASHIELD mounting solution was obtained from Vector Laboratories (Burlingame, CA, USA). U0126 was obtained from Calbiochem (Cambridge, MA, USA). XAV939 was purchased from Tocris Bioscience (Bristol, UK).

Cell line and cell cultures
Rat vibrissa immortalized DPCs were kindly provided by the Skin Research Institute of Amore Pacific Corporation R&D Center (Yongin, Korea). DPCs were cultured in DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin solution in a humidified atmosphere of 5% CO₂ at 37°C.

WST assay
DPCs (1.5×10⁴ cells/well) were seeded onto 96-well plates with DMEM containing 1% FBS. After 24 h, DPCs were treated with MA (1, 5, and 10 μM) or MXD (10 μM) for 48 h. To investigate whether ERK or Wnt/β-catenin signal pathway affects MA-induced DPC proliferation, DPCs were pre-treated with U0126 (ERK inhibitor, 10 μM) or XAV939 (Wnt/β-catenin inhibitor, 20 μM) for 30 min, and then treated with MA (5 μM) for 48 h. WST (10 μL/well) was added to medium and incubated for 2 h in a 5% CO₂ at 37°C. The absorbance was measured at 450 nm using a Versamark microplate reader ( Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis
DPCs (1.5×10⁵ cells/dish) were seeded in DMEM containing 1% FBS for 24 h and then treated with different concentrations (1 and 5 μM) of MA for 24 h. The cells were harvested, washed with PBS, fixed in 70% ethanol, and stored at −20°C for at least 30 min. After fixation, the cells were washed with PBS and stained with propidium iodide (PI, 50 μg/mL) in PBS containing 50 μg/ml RNase A for 30 min at 37°C. The cell cycle distribution was analyzed using a FACStar flow cytometer (BD)
Western blotting

DPCs (1.5×10⁶ cells/dish) were seeded in DMEM containing 1% FBS on 6-well plates with cover-glasses and stabilized for 24 h. To evaluate the translocation of β-catenin to the nucleus, cells were treated with different concentrations of MA (1 and 5 μM) or with MA (5 μM) in the absence or presence of XAV939 (20 μM) for 24 h. In addition, to confirm the LC3 puncta, DPCs were treated with inhibitor (U0126; 10 μM or XAV939; 20 μM) for 30 min, and then treated with 5 μM of MA for 24 h. For immunofluorescent staining, the cells were washed with cold PBS and fixed with 4% PFA for 10 min. After washing the cells with cold PBS three times, the cells were reacted with 0.5% Triton X-100 for 7 min for permeability. The cells were then blocked with primary antibody (anti-β-catenin (1:50), anti-LC3 (1:50), or anti-LC3 (1:50)) at 4°C overnight. The cells were washed, and then treated with 5 μM of MA for 24 h. For immunofluorescent staining, the cells were washed with cold PBS and stained with the corresponding AlexaFluor® 488 or 594-conjugated secondary antibody for 1 h at room temperature. After washing, the cells were mounted in Vectastain (Vector Laboratories) containing DAPI. Images were acquired using a confocal microscope (FluoView® FV1200; Olympus, Tokyo, Japan).

Statistical analysis

All experimental data are denoted as the mean ± standard deviation (SD) from three independent experiments. A p-value less than 0.05 in the two-tailed Student’s t-test was considered significant.
significant.

RESULTS

MA stimulates the proliferation of DPCs

To determine whether MA affects the proliferation of hair follicle cells, DPCs were treated with MA at different concentrations (1, 5, and 10 μM) for 48 h, followed by a WST assay. As a result, MA treatment was found to enhance the proliferation of DPCs by 106.9% ± 1.0% (p < 0.01), 107.4% ± 0.2% (p < 0.001), and 107.1% ± 7.2% at 1, 5, and 10 μM, respectively (Fig. 2A). MXD 10 μM, which was used as the positive control, was also found to significantly increase the proliferation of DPCs (106.5% ± 0.5%; p < 0.01) (Fig. 1B). Cell division regulated by cell cycle progression is a necessary process for cell proliferation, defined as an increase in the cell number (Golias et al., 2004). In order to determine whether DPC proliferation results from changes in the cell cycle by MA, the cell cycle distribution and the expression of cell cycle-related proteins were quantified using a flow cytometer and western blotting, respectively. As shown in Fig. 1C, compared to DMSO-treated control group, MA treatment for 24 h increased the cell population of G2/M phase in DPCs. Moreover, MA increased the levels of proteins involved in the progression of G2/M phase, such as cyclin A, Cdc2, and cyclin B1 (Fig. 1D). These results suggest that MA induces the proliferation of DPCs by regulating the progression of the G2/M phase cell cycle.

MA induces autophagy in DPCs

Autophagy plays an important role in hair growth by maintaining the anagen phase during the hair cycle (Parodi et al., 2018). Therefore, we investigated whether MA induced autophagy in DPCs. The activation of mTOR regulates cell proliferation by participating in multiple pathways, and is also involved in the initiation of autophagy and the formation of autophagosome (Zou et al., 2020). The conversion of LC3I (free form) to LC3II (lipid-conjugated form) is a major step in autophagosome formation. Lipid conjugation, during which LC3I forms LC3II by binding with phosphatidylethanolamine on the surface of autophagosome, is caused by the action of Atg7 and Atg3 (Frudde et al., 2018). When DPCs were exposed to MA 5 μM for 0.5-6 h, MA inhibited mTOR phosphorylation. In addition, the expression of Atg7 and LC3II by MA treatment increased from 0.5 and 1 h, respectively (Fig. 2A). To examine the effective concentrations of MA for autophagy induction, DPCs were treated with 1 or 5 μM of MA for 24 h. The levels of Atg7 and LC3II significantly increased by 5 μM of MA compared to the DMSO-treated control group (Fig. 2B). Since the amount of LC3II is correlated with the number of autophagosomes (Yoshii and Mizushima, 2017), we evaluated the accumulation of autophagosomes using fluorescence microscopy with LC3II antibody. As shown Fig. 2C, MA enhanced the formation of LC3 puncta in DPCs. When quantifying the number of LC3 puncta in the fluorescence microscopy results, MA increased the number of LC3 puncta in a dose-dependent manner (Fig. 2C). This indicates that MA activates autophagy in DPCs.

MA activates Wnt/β-catenin signaling in DPCs

The Wnt/β-catenin pathway regulates cell proliferation, and is essential for the hair cycle, hair morphogenesis, and hair regeneration (Ito et al., 2007). MA was reported to enhance Wnt reporter activity in DPCs (Lee et al., 2016). However, whether MA regulates the activation and nuclear translocation of proteins in the Wnt/β-catenin pathway is yet to be deter-
MA promotes autophagy and cell cycle progression by activating of ERK pathway

Next, we evaluated whether MA activates ERK pathway. ERK is a signal transduction pathway that regulates the cell cycle, autophagy, and proliferation of many cells (Chambard et al., 2007; Sun et al., 2015; Fung and Liu, 2019). MA treatment was found to increase the levels of phospho (Thr202/Tyr204)-ERK after 15-30 min (Fig. 5A). Thus, we investigated whether ERK activation affects cell cycle progression and autophagy. U0126, an ERK inhibitor, attenuated the increase of MA-induced ERK phosphorylation (Fig. 5B). U0126 pretreatment attenuated the MA-induced increase in the levels of Cdc2 and Atg7, an autophagy-related protein, while XAV939 treatment attenuated the MA-induced increase in the levels of these proteins (Fig. 4B). In addition, when DPCs were pre-treated with XAV939 for 30 min, XAV939 attenuated the MA-induced LC3 puncta (Fig. 4C). Moreover, XAV939 significantly reduced MA-induced DPC proliferation, while only XAV939 treatment did not affect DPC proliferation (Fig. 4D). The results using XAV939, a Wnt/β-catenin signaling inhibitor, suggest that MA stimulated cell-cycle progression and autophagy via the activation of the Wnt/β-catenin pathway in DPCs, which was followed by an increased DPC proliferation.

**Fig. 3.** MA activates the Wnt/β-catenin pathway in DPCs. The effects of MA on the levels of Wnt/β-catenin pathway-related proteins. (A) DPCs were treated with MA (5 μM) or DMSO for the indicated times (15 and 30 min), and western blotting was performed with anti-phospho (Ser47) -GSK3β, -phospho (Ser32) -β-catenin, -phospho (Ser25) -β-catenin, -phospho (Ser215) -β-catenin, and -β-catenin. -actin was used as the loading control. (B) DPCs were stimulated with MA (1 and 5 μM) for 24 h, and the protein levels were examined by western blotting with the corresponding antibodies. (C) Immunofluorescent staining was performed to confirm the localization of β-catenin. DPCs were stained with MA (5 μM) for 24 h. The DPCs were stained with anti-β-catenin (1:50) and -α-tubulin (1:50). Anti-AlexaFluor-594 (1:200) and -AlexaFluor-488 (1:200) secondary antibodies were used to detect β-catenin and α-tubulin, respectively. DAPI was used to stain the nuclei. Images were obtained using a Fluoview® FV1200 confocal microscope (40× objective). Scale bar, 50 μm.
**DISCUSSION**

The present study showed that MA enhances anagen signaling by autophagy and G2/M phase cell cycle progression through the activation of the Wnt/β-catenin and ERK pathways in DPCs. Although the regulatory mechanisms underlying hair growth are not yet fully understood, hair growth is known to be regulated by the interactions between DPCs and various types of cells, including keratinocytes, hair germ cells, and stem cells (Stenn and Paus, 2001). In particular, the interactions between DPCs and keratinocytes, which are known to be important for the regulation of both the hair cycle and hair growth (Sennett and Rendl, 2012). However, it has been reported that different sizes and types of hair are generated if the number of keratinocyte stem pools (Chi et al., 2013). Therefore, among follicular cells, DPCs play an essential role in hair growth, determining the size and shape of the hair follicle, as well as maintaining the anagen phase (Chi et al., 2013). In general, cell proliferation is associated with cell cycle progression. Cell cycle progression is regulated by the interactions between various proteins, such as cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CDKIs) (Malumbres and Barbacid, 2009). In this study, MA was found to increase the proliferation of DPCs and the population of G2/M phase in the cell cycle (Fig. 1B-1D). In the G2- and M-phases, the interactions between cyclin A/Cdc2 (CDK1) and cyclin B/Cdc2 is important. As shown in Fig. 1D, MA increased the levels of G2/M phase-related proteins, including cyclin A, Cdc2, and cyclinB1.

There are many studies related to hair growth and regeneration that control the microenvironment. However, little is known regarding the regulation of intracellular metabolic pathways (Chai et al., 2019). mTOR is a serine/threonine protein kinase of the PI3K-related kinase (PIKK) family that has many roles in the regulation of cell metabolism, growth, proliferation, cell cycle progression, and protein synthesis. mTOR is known to play an important role in various diseases, such as diabetes and cancer (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). However, studies on the role of mTOR in hair growth and regeneration are still lacking and often controversial. Several studies have reported that mTOR is required for the activation of hair follicle stem cells and anagen entry (Castilho et al., 2009). However, another study found that hair growth at
the anagen phase is induced by rapamycin, an mTOR inhibitor (Chai et al., 2019). As shown in Fig. 2A, MA inhibited mTOR phosphorylation. In addition, mTOR, a negative regulator of autophagy, is a key molecule for the initiation of autophagy (Shi et al., 2019).

Autophagy is an essential cellular process tasked with maintaining homeostasis against various stresses by breaking down damaged or unfolded proteins and aged organelles. In the process of autophagy, early or initial autophagic vacuoles (AVi, autophagosome) and late or degradative autophagic vacuoles (AVd, autolysosome) are formed. The autophagosome is a double membrane structure that separates cytoplasmic material via autophagy initiation signals. It fuses with lysosomes to form autolysosomes, which then decomposes internal materials (Kraft and Martens, 2012). The importance of autophagy for the self-renewal and differentiation of epidermal and dermal stem cells in the skin has already been demonstrated (Belleudi et al., 2014). However, the role of autophagy in the regulation of hair growth, regeneration, and follicle stem cells has yet to be fully elucidated.

In the organ culture model of human scalp hair follicles, the volume of autophagy-related structures increased in the anagen phase compared to the catagen phase, suggesting that autophagy is essential for the maintenance of the anagen phase (Parodi et al., 2018). In addition, skin grafts obtained from mice deficient in Atg7, which is important for autophagosome formation, exhibited abnormal hair growth (slower hair growth compared to the control group) (Yoshihara et al., 2015). Recent studies have also demonstrated the existence of a relationship between autophagy and bone morphogenetic protein (BMP)-2, which is important for the differentiation of hair follicle stem cells (Cai et al., 2019). Furthermore, small molecules, such as a-ketoglutarate (a metabolite), oligomycin (a complex V inhibitor), 5-aminimidazole-4-carboxamide ribonucleotide (an AMPK activator), metformin (an AMPK agonist), and rapamycin (a mTOR inhibitor), have been reported to be involved in metabolism and autophagy induction, as well as hair growth regulation (Chai et al., 2019). In this study, MA was found to increase the levels of Atg7 and LC3II in DPCs in both a time- and dose-dependent manner (Fig. 2A, 2B). Moreover, the number of LC3 puncta increased after treatment with MA (5 μM) or DMSO for the indicated times (15 and 30 min), followed by western blotting using anti-phospho (Thr217/Tyr221)-ERK and -ERK, β-actin was used as the loading control. (B) Immunofluorescent staining was performed with anti-LC3II (1:200). (C) Western blotting was performed with anti-Cdc2, -Atg7, and -β-actin. (D) Immunofluorescent staining was performed using anti-phospho (Thr217/Tyr221)-ERK, ERK, and β-actin levels were analyzed using western blotting. (C, D) DPCs were treated with MA (5 μM) in the presence or absence of U0126 (10 μM) for 24 h. (E) DPCs were pretreated with MA (5 μM) for 30 min, followed by exposure to MA (5 μM). The proliferation of DPCs was measured by WST assay 48 h after treatment. Data are shown as the mean of three independent experiments. Error bars represent SD. *p<0.05, **p<0.01, and ***p<0.001.
tion, and hair growth (Ito et al., 2007). However, the regulation of autophagy by the Wnt/β-catenin pathway is not well known and remains controversial. Several studies have reported that β-catenin signaling acts as a negative regulator of autophagy (Su et al., 2016), while others have reported that autophagy is induced by β-catenin (Fan et al., 2018). Thus, we investigated whether the induction of autophagy and the proliferation of DPCs by MA were related to the Wnt/β-catenin pathway. The stability of β-catenin is regulated by the phosphorylation of GSK3β. When the serine 33 and 37 residues of β-catenin are phosphorylated by GSK3β, β-catenin is degraded in the cytosol via the proteasome pathway. In addition, when β-catenin is stabilized by the phosphorylation of the serine 552 and 675 residues, β-catenin is translocated to the nucleus for the transcription of the target genes (Spirli et al., 2013). As shown in Fig. 3, MA increased phospho (Ser9)-GSK3β via the proteasome pathway. In addition, when phosphorlyated by GSK3β, GSK3β residues, transcription of the target genes (Spirli et al., 2013).

Our results demonstrate that MA stimulated the ERK pathway, growth factors, such as vascular endothelial growth factor (VEGF), and has recently been reported to regulate autophagy (Peng et al., 2017). In the present study, XAV939 was found to decrease the MA-induced nuclear β-catenin levels, followed by a reduction of MA-induced autophagy and cell proliferation (Fig. 4). ERK is a signaling mechanisms of mitogen-activated protein kinases (MAPKs) and is involved in cell proliferation, survival, differentiation, adhesion, and migration (Busca et al., 2016). The ERK pathway is known to control the proliferation of many cells (Sun et al., 2015), including DPCs (Huang et al., 2019), through various mechanisms, such as cell cycle and autophagy regulation (Chambard et al., 2007; Chen et al., 2018). In addition, MXD, various herbal extracts, flavonoids, natural compounds, and growth factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), stimulate hair growth via the regulation of the ERK pathway in DPCs (Choi et al., 2018; Madaan et al., 2018). Our results demonstrate that MA stimulated the ERK pathway, while the blocking of the ERK pathway was found to inhibit cell cycle progression, autophagy, and cell proliferation in DPCs (Fig. 5).

In conclusion, MA appears to promote DPC proliferation by inducing cell cycle progression and autophagy via the Wnt/β-catenin and ERK pathways. Our study provides scientific evidence for the applicability of MA as a treatment that could potentially alleviate hair loss.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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