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Hot Water Extract of Oriental Melon Leaf Promotes Hair Growth and Prolongs Anagen Hair Cycle: *In vivo* and *In vitro* Evaluation

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Abstract Effects of hot water extracts of oriental melon leaves (OML) on promotion of hair growth were investigated. Topical OML extract administration for hair regeneration was investigated using an *in vivo* model with C57BL/6 mice. Effects of OML extracts on the human hair growth were investigated using a hair follicle organ culture. OML extracts induced a shortened telogen to anagen conversion and promoted hair growth in the C57BL/6 mouse model. Culture of human hair follicles in the presence of OML extracts for 8 days promoted hair growth and prolonged the anagen duration due to induction of hair follicle cell proliferation in the bulb region. OML extracts exert a hair growth promotion effect and, therefore, can be used as a therapeutic agent for prevention of hair loss.

Keywords: oriental melon leaf, C57BL/6 mouse model, hair follicle organ culture, hair growth, hair cycle

Introduction

Hair follicles are complex skin appendages that produce hair. During postnatal life, hair cyclically undergoes the 3 alternating phases of rapid growth and hair production (anagen), apoptosis-mediated regression (catagen), and relative quiescence (telogen) (1). Dysregulation of the hair cycle has been shown to be associated with pathogenesis of androgenetic alopecia (AGA), among other conditions (2-5).

AGA is the most common hair loss disorder affecting both men and women. The key feature of AGA is progressive shortening of anagen duration and a resultant progressive miniaturization of terminal hairs to vellus-like hairs (6,7). Therefore, the ability to prolong or promote anagen duration is considered to be a key element in development of treatments for and prevention of AGA. Minoxidil and finasteride are drugs that have been approved by the Food and Drug Administration (FDA) for AGA treatment (8,9). However, the fact that 20-30% of AGA patients receiving these drugs are non-responders provides motivation to search for alternative therapeutics. Many attempts at AGA treatment have focused on identification of effective hair loss remedies using traditional herbal medicines (10-17).

The oriental melon (*Cucumis melo* var. *makuwa*), called chamoe in Korea, is a popular fruit crop cultivated mainly in Asia (18). In Korean folk medicine, chamoe has been used to treat acute gastritis, fever, mental disorders, dysuria, jaundice, alcoholism, and hyperesthesia/paralysis (19). Oriental melon leaves (OML) have been reported to exert anti-microbial, anti-oxidative, and chemopreventive effects (20-22). However, effects of OML on hair growth have not yet been reported.

This study was conducted for evaluation of OML hot water extract effects on promotion of hair growth. Effects of topical OML extracts on hair regeneration were investigated in an *in vivo* model using C57BL/6 mice. Furthermore, effects of OML extracts on human hair growth were investigated using a hair follicle organ culture.

Materials and Methods

Materials Reagents used in this study were Minoxidil (5%, MINOXYL^{TM}) (Hyundai Pharm. Co., Ltd., Chunan, Korea), hematoxylin (BBC Biochemical, Mount Vernon, WA, USA), eosin (Shimakyu's Pure Chemicals, Osaka, Japan), Dulbecco's phosphate buffered saline (Hyclone, Logan, UT, USA), Williams E medium (Invitrogen, Gaithersburg, MD, USA), insulin (Sigma-Aldrich, St. Louis, MO, USA), L-glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), and the Ki-67 antibody (Abcam, Cambridge, MA, USA).

Preparation of OML hot water extracts A pilot screening procedure using OML hot water, methanol, and ethanol extracts in an *in vivo* model of C57BL/6 mice was performed. Hot water OML extracts showed a marked effect for promotion of hair growth, compared with other extraction methods. Consequently, 100 g of OML was

extracted using 1,000 mL of hot water under reflux for 120 min at 100°C. Hot water extracts were evaporated using a rotary evaporator, then recovered using a freeze dryer. The resulting residue was subsequently stored at −20°C prior to dissolution in water for experimental use.

Animal studies All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University, Wonju College of Medicine (Certified protocol number: YWC-140702-1). Female C57BL/6 mice (6 weeks of age) were purchased from Orient Bio (Seongnam, Korea) and provided with a standard laboratory diet and water *ad libitum*. Anagen was induced based on depilation of the dorsal skin of C57BL/6 mice that were in the telogen phase, as described previously (23). Briefly, 6-week-old female C57BL/6 mice were environmentally adapted to the new environment for a week. Anagen was then induced using depilation of the dorsal skin of 7-week-old female C57BL/6 mice, which led to synchronized development of anagen hair follicles (23). Beginning the following day, 0.2 mL of Dulbecco's phosphate buffered saline (DPBS) (vehicle, *n*=6) and 0.2 mL of a 3 mg/mL OML extract dissolved in DPBS (*n*=6), were applied topically every day for 28 days to the depilated area with 5% Minoxidil (Hyundai Pharm.) used as a positive control (*n*=6). The depilated dorsal area of each mouse was then observed and photographed at 0, 7, 14, 21, and 28 days after depilation. A hair growth score was applied to each mouse as: no hair growth=0, less than 20% hair growth=1, 20% to 40% hair growth=2, 40% to 60% hair growth=3, 60% to 80% hair growth=4, and 80% to 100% hair growth=5 (15).

Hematoxylin and eosin (H&E) staining Mouse dorsal skin collected from each group on treatment days 0, 7, 14, and 28 was fixed in 10% buffered formaldehyde at pH 7 and embedded in paraffin. Serial sections of 4 μm were cut and mounted on slides. Sections were deparaffinized using xylene, hydrated in a descending series of graded ethanol, and stained with hematoxylin for 2 min, followed by washing for 2 min and eosin staining for 5 s.

Isolation and culture of human hair follicles Human occipital scalp skin samples were obtained from hair transplantation surgery patients after informed consent. A total of 6 donor samples were obtained from 4 different individuals. The Institutional Ethics Committee of Yonsei University Wonju College of Medicine, Wonju, Korea, approved all of the described studies, which were conducted according to the Declaration of Helsinki. Human hair follicles in anagen stage VI were isolated as previously described (24). Briefly, after separation of the epidermis and dermis from the dermo-subcutaneous interface, anagen hair follicles were isolated from subcutaneous fat under a light microscope using watchmaker forceps and maintained in Williams E medium (Invitrogen) supplemented with 10 μg/mL insulin (Sigma-Aldrich), 10 ng/mL hydrocortisone (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), 100 IU/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen). Hair follicles were maintained free-floating at 37°C in an atmosphere of 5% CO_2 and 95% air in a humidified incubator. Hair follicles were treated with 10 or 100 μg/mL OML extracts, and 1 μM minoxidil (Sigma-Aldrich) was used as a positive control.

Measurement of hair follicle length and morphology Hair follicle length was defined as the entire length from the base of the hair follicle bulb to the tip of the hair shaft using a measuring scale attached to a microscope objective lens. Measurements were taken at 2-day intervals until the 8th day of cultivation and resulting data were subjected to statistical analysis. At the same time, hair follicle morphology (anagen, early catagen, mid catagen, and late catagen) was observed and assigned a hair cycle score (HCS) as: anagen VI= 100, early catagen=200, mid-catagen=300, and late catagen=400 (23,25,26).

Immunofluorescence staining After deparaffinization and rehydration, sections were fixed using 4% paraformaldehyde (Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing 0.1% Triton X-100 (Sigma-Aldrich) for 10 min and equilibrated in phosphate-buffered saline (Gibco BRL, Gaithersburg, MD, USA) for 15 min at room temperature for immunofluorescence staining of Ki-67. After blocking using 4% normal donkey serum, sections were incubated with mouse monoclonal Ki-67 antibody (Abcam) and incubated with Alexa Flour 488 labeled donkey anti-mouse secondary antibody (Abcam). Sections were then counterstained with propidium iodide (PI) (Sigma-Aldrich) and visualized using a Leica TCS-SPE confocal microscope (Leica Microsystems, Bannockburn, IL, USA). The number of cells positive for Ki-67 immunoreactivity was counted.

Statistical analysis Data handling and drawing were processed using the SPSS ver. 20.0 (SPSS Inc., Chicago, IL, USA) for Windows statistical package. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for comparison of 3 or more groups. Student's *t*-test was used for comparison of 2 different groups. Significance was defined as $p<0.05$. All data were reported as a mean±standard error of the mean (SEM) of at least 3 separate experiments.

Results and Discussion

Effects of OML extracts on anagen induction in C57BL/6 mice Hair growth-promotion activities of OML extracts were investigated using depilated 7-week-old C57BL/6 mice, as described previously (23,26).

After synchronization of the hair cycle with depilation and all hair follicles in the telogen phase, effects of topical OML extract administration on hair regeneration were investigated. Mice receiving 5% minoxidil and 3 mg/mL OML extracts showed post-hair growth induction, with a visible hair shaft after 7 days of application, while skin of control

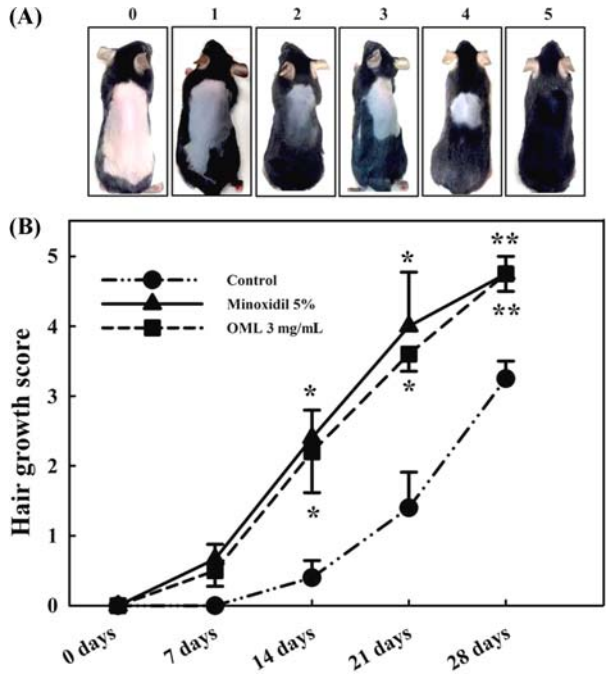

Fig. 1. Effects of OML extracts on hair growth in C57BL/6 mice. (A) Hair growth scores were assigned to mice according to the following system: no hair growth, 0; less than 20% hair growth, 1; 20% to 40% hair growth, 2; 40% to 60% hair growth, 3; 60% to 80% hair growth, 4; 80 to 100% hair growth, 5. (B) Quantitative analysis of hair growth scores after treatment with OML extracts. Data are based on 6 mice per group and are reported as a mean±SEM. *p<0.05, compared with vehicle-treated controls.

mice remained pink after 7 days (Fig. 1). OML extract-treated mice showed remarkable promotion of hair growth after 14 days of application. Hematoxylin and eosin staining was performed for investigation of progression of the hair cycle. At day 7, hair follicles for both OML extract and minoxidil-treated mice had progressed to the anagen phase, whereas hair follicles in control mice remained in the telogen phase (Fig. 2). At day 14, hair follicles in control mice had progressed to the anagen phase, whereas OML extract and minoxidil-treated mice showed markedly increased depth and size of hair follicles, compared with control mice (Fig. 2). At day 28, most hair follicles in mice of each treatment group were in the anagen VI stage (Fig. 2). Thus, OML extracts induced an earlier telogen-to-anagen conversion and prolonged the mature anagen phase in C57BL/6 mice.

Effects of OML extracts on hair growth in a human hair follicle organ culture A hair follicle culture system has been shown to be a powerful model for determination of direct effects of enzyme inhibitors, substrate deletions, and product additions on follicular function (24,27,28). Hair growth promotion effects *in vivo* of OML extracts were also observed in a human hair follicle organ culture.

Human hair follicles were treated with 10 or 100 μg/mL OML extracts, or 1 μM minoxidil as a positive control, for 8 days. Mice treated with 100 μg/mL OML extracts showed significantly increased hair follicle lengths after 4 days, compared with control mice (Fig. 3). In addition, mice treated with 1 μM minoxidil also showed significantly

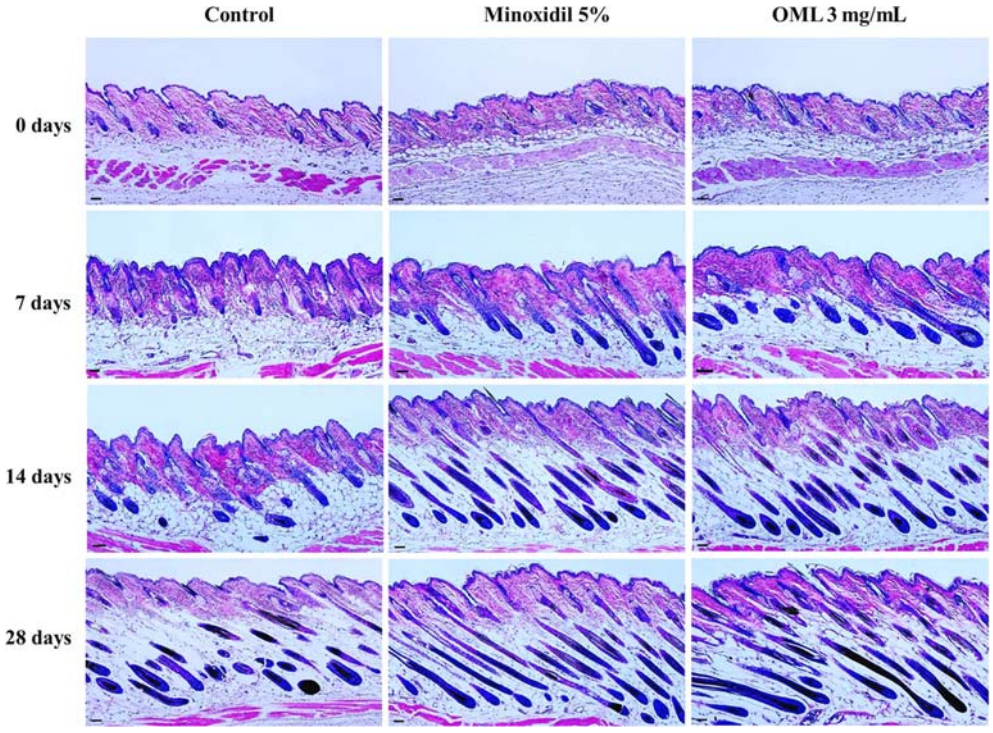

Fig. 2. Histological analysis. Dorsal skin of mice stained with hematoxylin and eosin at 0, 7, 14, and 28 days after depilation (Bar=0.1 mm).

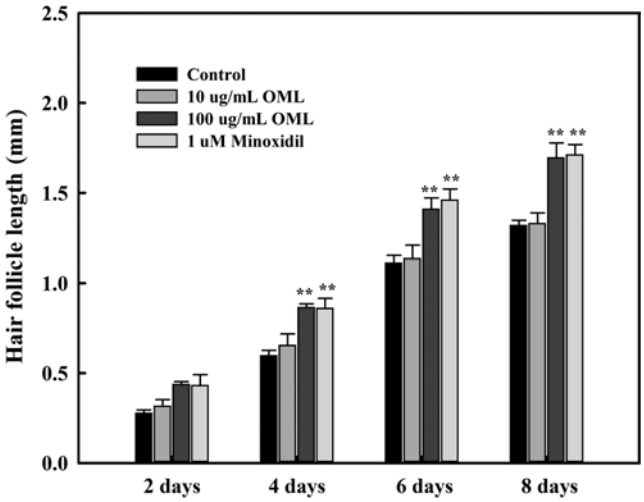

Fig. 3. Quantitative analysis of hair growth after treatment with OML extracts. Hair follicle length was measured at 2-day intervals. Data are based on 12 hair follicles per group and are reported as a mean±SEM. ***p*<0.01, compared with vehicle-treated controls.

increased hair growth after 4 days, compared with control mice (Fig. 3). Thus, OML extracts directly exerted a growth-modulation effect on hair follicles.

Effects of OML extracts on anagen duration in a human hair follicle organ culture Treatment with OML extracts was investigated for changes in hair cycling assessed based on quantitative hair cycle histomorphometry, as described previously (25). After 4 days of organ culture, OML extracts dose-dependently decreased HCS values (10 μg/mL OML extract=155.556±24.216, 100 μg/mL OML extract= 133.333±16.667), compared with vehicle treated controls (166.667± 23.570) (Fig. 4). After 8 days of organ culture, 100 μg/mL OML extract and 1 μM minoxidil-treated hair follicles showed markedly decreased HCS values (100 μg/mL OML extract=166.667±23.570, 1 μM minoxidil=155.556±24.216), compared with vehicle treated controls (288.889±26.058). Thus, catagen progression in OML extract-treated hair follicles was delayed.

Effects of OML extracts on cell proliferation of hair follicles Proliferation of hair follicle bulb keratinocytes is an important event for anagen hair growth (2). Activated hair follicle bulb keratinocytes are rapidly proliferating cells, the number of which determines the size of the hair follicle bulb and the diameter of the hair shaft (29). For evaluation of OML extract effects on cell proliferation of hair follicles, immunofluorescence staining for Ki-67 was performed as a proliferation marker. Both 100 μg/mL OML extract and 1 μM minoxidil-treated hair follicles exhibited markedly increased numbers

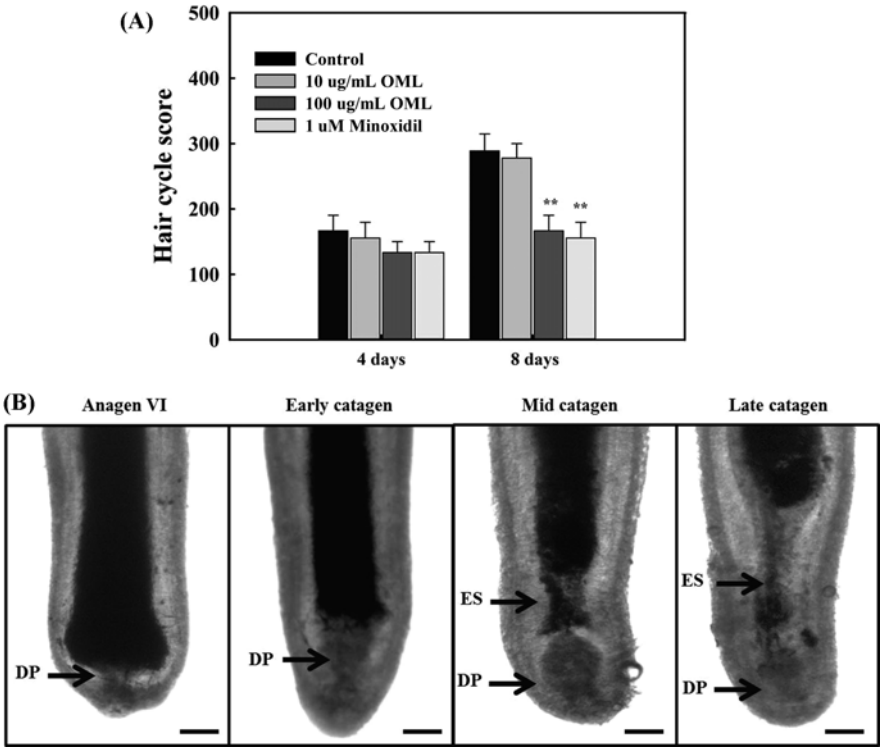

Fig. 4. Hair cycle scores after treatment with OML extracts. (A) Hair follicles of each group were staged and scored as follows: anagen VI, 100; early catagen, 200; mid-catagen, 300; late catagen, 400. (B) Hair cycle stage of each hair follicle was assessed and classified as previously described (23). Anagen VI fully developed terminative hair follicles show a prominent onion-shaped hair bulb and a narrow and elongated dermal papilla. Early catagen phase follicles show a narrow hair bulb fully opened at the proximal end. Mid-catagen phase follicles show a partially keratinized presumptive club just above the dermal papilla. Late catagen phase follicles show a narrower epithelial strand. Data are based on 9 hair follicles per group and are reported as a mean±SEM. ***p*<0.01, compared with vehicle controls. Bar=0.1 mm. DP, dermal papilla; ES: epithelial strand.

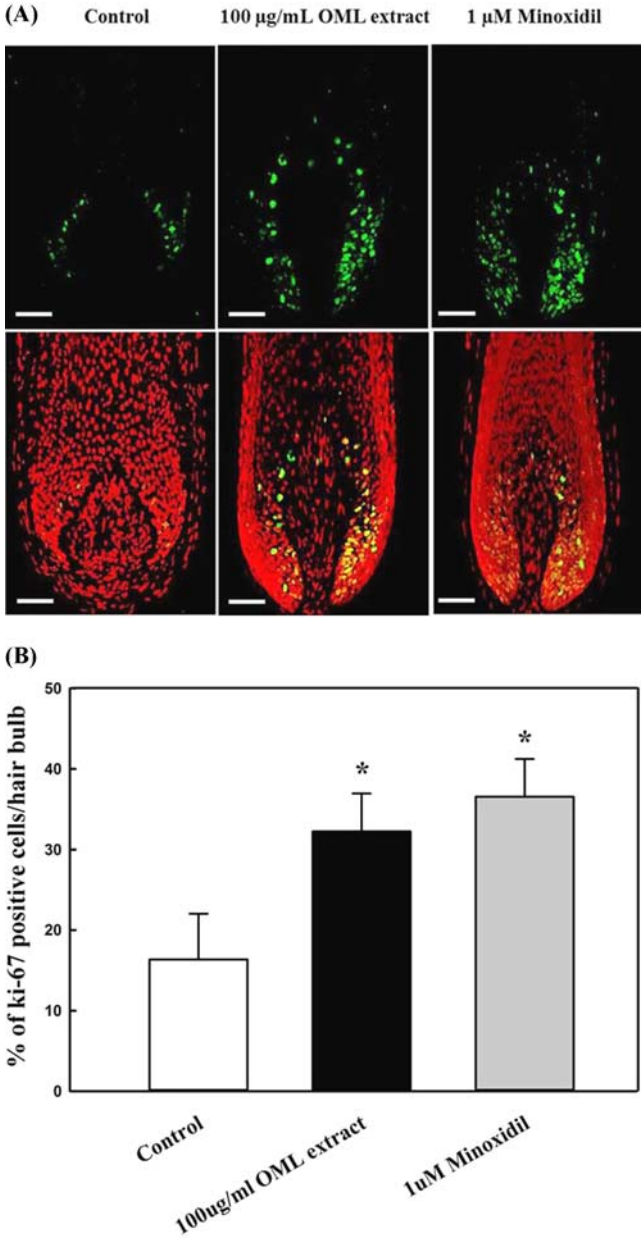

Fig. 5. Analysis of Ki-67-positive cells in human hair follicles after treatment with OML extracts. Human hair follicles were treated with OML extracts or minoxidil for 4 days. (A) Sections of hair follicles were analyzed for proliferation (Ki-67-positive, green fluorescence) of hair follicle bulb keratinocytes. Nuclei were counterstained with propidium iodide (PI, red fluorescence). (B) For quantitative analysis, the number of Ki-67 positive cells was counted and normalized to the number of PI-stained cells in hair follicle bulb keratinocytes. Data are based on 6 hair follicles per group and are reported as a mean±SEM. *p<0.05, compared with vehicle controls. Bar=0.1 mm.

of Ki-67 positive keratinocytes, compared with vehicle treated controls (Fig. 5), indicating that OML induced proliferation of hair bulb keratinocytes.

In conclusion, this study was conducted for evaluation of hair growth promotion effects of hot water extracts of OML. OML extracts induced a shortened telogen to anagen conversion and promoted hair growth in a C57BL/6 mouse model. Culture of human hair follicles in the presence of OML extracts for 8 days promoted hair growth and prolonged the anagen duration based on induction of hair follicle cell proliferation in the bulb region. OML extracts exerted a hair growth promotion effect and, therefore, can serve as a therapeutic agent for prevention of hair loss. Further detailed clinical trials and studies for documentation of efficacious components and ingredients of hot water OML extracts should proceed. This study used whole OML extracts rather than individual component because the study objective was verification that OML is a potent functional medicinal source for treatment of pathogenic alopecia.

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Disclosure The authors declare no conflict of interest.

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