Antimelanogenesis Effects of Fungal Exopolysaccharides Prepared from Submerged Culture of *Fomitopsis castanea* Mycelia^S

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Fungal exopolysaccharides are important natural products having diverse biological functions. In this study, exopolysaccharides from *Fomitopsis castanea* mycelia (FEPS) were prepared, and the highest mushroom tyrosinase inhibitory activity was found. FEPS were prepared from cultivation broth by ethanol precipitation method. The extraction yield and protein concentration of FEPS were 213.1 mg/l and 0.03%, respectively. FEPS inhibited mushroom tyrosinase with the half maximal inhibitory concentration (IC₅₀) of 16.5 mg/ml and dose-dependently inhibited cellular tyrosinase activity (63.9% at 50 µg/ml, and 83.3% at 100 µg/ml) in the cell-free extract of SK-MEL-5 human melanoma cell and α -melanocyte-stimulating hormone (α -MSH)-stimulated melanin formation in intact SK-MEL-5 human melanoma cell. The IC₅₀ of FEPS against NO production from RAW264.7 macrophage cells was 42.8 ± 0.64 µg/ml. By in vivo study using a zebrafish model, exposure of FEPS at 400 µg/ml to dechorionated zebrafish embryos for 18 h decreased the pigment density, compared to that without FEPS-treated control.

Keywords: *Fomitopsis castanea*, exopolysaccharides, SK-MEL-5 human melanoma cells, zebrafish, tyrosinase

Introduction

Mushrooms are known as functional foods because of their bioactive compounds that are considered beneficial for human health [1]. The biological and pharmaceutical activities of mushroom are due to cellular and secondary metabolite compounds, which have been extracted from the fruiting body and cultured mushroom medium [1]. Exopolysaccharides (EPS) are secondary metabolite polymers that are secreted into the culture medium by microorganism during the growth process. EPS are used in foods, pharmaceuticals, biomedical, and cosmetic industries because of their biological and pharmaceutical activities such as tyrosinase inhibitory activity [2], hepatoprotective effect, antioxidant activity, immunomodulation property [3, 4], anti-inflammatory [5], antitumor, antidiabetic, antimicrobial, and/or immune-stimulatory activities [6] and

prebiotic effect [7]. EPS are composed of repeating units of monosaccharides joined by glycosidic linkages [8]. The structure of EPS varies in different species, due to the different of monosaccharides and glycosyl linkages in their repeating unit. These differences in EPS structure affect their biological function [9, 10]. The fungal EPS are easier to isolate, purify, and cheaper to produce in vast quantity and less time compared to EPS prepared from fruiting bodies [9, 10].

Melanogenesis is the process for the production of melanin, enhanced by melanogenic enzymes such as tyrosinase and related proteins, for protecting the skin from UV irradiation [11, 12]. However, overproduction of melanin can cause aesthetic problems [13]. Tyrosinase catalyzes the hydroxylation of L-tyrosine and the oxidation of 3,4-dihydroxy-L-phenylalanine (L-DOPA) [14], and then the polymerization of pigments [15]. Thus, tyrosinase

inhibitors can be used as skin-brightening agents in cosmeceuticals [11, 16].

Fomitopsis castanea is a type of wood-decay medicinal fungi species [17]. The purification and characterization of EPS from *F. castanea* have been reported [17]. That study focused on the determination of EPS structure and in vitro fermentation of EPS in a simulated intestinal environment. The main monosaccharides of EPS from *F. castanea* were arabinose, mannose, rhamnose, galactose, and glucose [17]. Adding EPS to the fermentation system of the simulated intestinal environment increased the production of short-chain fatty acid content in the fecal extract of tested human [17]. However, there has been no report of the melanogenesis inhibitory effects and anti-inflammatory activity of EPS from *F. castanea*. In this regard, we isolated EPS from submerged culture of *F. castanea* mycelia (FEPS) and found that FEPS inhibited melanin biosynthesis and tyrosinase activity. Furthermore, using SK-MEL-5 human melanoma cells and zebrafish, the FEPS inhibited melanogenesis and tyrosinase activity both in vitro and in vivo, respectively. Therefore, FEPS have the potential to be used as a natural, functional tyrosinase inhibitor in cosmetic application.

Materials and Methods

Screening of Exopolysaccharides from Different Fungal Species against Mushroom Tyrosinase

The lyophilized culture supernatants from *Abortiporus biennis*, *Cryptoporus volvatus*, *Fomitopsis castanea*, *Phallus luteus*, *Pholiota alnicola*, and *Pholiota limonella* mycelia were obtained from the Korea National Institute of Biological Resources (NIBR, Korea). The crude EPS from 6 fungal species were prepared by adding 400 µl chilled ethanol to 8 mg of cell-free supernatant powder for overnight at 4°C. Samples were then centrifuged at 3,200 ×g for 30 min at 4°C. The protein was removed using the Sevag method [18]. Briefly, chloroform and n-butyl alcohol (4:1, v/v) were added to exopolysaccharides, then the mixture was vigorously mixed at vortices for 30 min, and centrifuged at 5,700 ×g for 20 min. The supernatants were carefully transferred to new Eppendorf tubes. The inhibitory effect of EPS against mushroom tyrosinase was carried out using reaction mixture containing 10 U/ml mushroom tyrosinase (Sigma, USA), 3.3 mM L-DOPA (Sigma), and 10% of fungal EPS in 50 mM potassium phosphate buffer (pH 6.8) for 10 min at 25°C. The absorbance of the reaction was measured at 475 nm using a SpectroMax M3 microplate reader (Molecular Devices, USA).

Preparation of Exopolysaccharides from *F. castanea* Using Submerged Culture Fermentation

F. castanea was grown on potato dextrose agar (PDA) medium in a Petri dish at 25°C for 7 days. Then, *F. castanea* was transferred

to the seed culture broth, by punching out 5 mm of the agar culture using a sterilized cork borer. The seed culture was grown in 200 ml DY media containing 2% (w/v) dextrose and 0.2% (w/v) yeast extract in a 500 ml flask at 25°C at 150 rpm for 7 days. Next, 10% (v/v) of the seed culture was transferred into 1 L culture media in a 1.6 L stirred-tank fermenter (Hanil Inc., Korea) at 25°C and 150 rpm for 18 days. The culture broth from the fermenter was filtered through 110 mm diameter filter paper (Whatman No. 5, Camlab., UK). The culture filtrate was then precipitated with four volumes of cold absolute ethanol, stirred vigorously, and kept overnight at 4°C. After centrifugation at 3,369 ×g for 30 min at 4°C, the precipitated EPS was dissolved in water, and then deproteinized by the Sevag method. The deproteinized polysaccharides were lyophilized at -10°C under 10 Pa (Eyela FD-550, Japan), and stored at -20°C for further study. The extraction yield of FEPS was calculated by weighing lyophilized precipitates centrifuged from cell-free culture [19]. The protein concentration in FEPS was determined by using Bradford assay with bovine serum (BSA) as standard. Briefly, samples or BSA standards (0.1 – 1.0 mg/ml) were added to a 96-well plate containing 200 µl of dye reagent (Bio-Rad Protein Assay kit II, Bio-Rad, USA) and mixed for 2 min. After 5 min at room temperature, the absorbance was read at 595 nm using a SpectraMax M3.

Identification of Monosaccharides of Exopolysaccharide

EPS (1 mg) was hydrolyzed using 0.5 M hydrochloric acid at 121°C for 30 min in a vial for the quantification and identification of monosaccharides. The monosaccharide composition of EPS was determined by thin layer chromatography (TLC). One µl of acid hydrolysate was spotted on a precoated silica gel 60 F₂₅₄ TLC (Merck, Germany) with glucose, galactose, mannose, rhamnose, and arabinose as standards [17]. The TLC plates were developed five times with acetonitrile: water (85:15, v/v) solvent system. The carbohydrates were then visualized by dipping the plates into a solvent mixture of 0.3 % (w/v) N-(1-Naphthyl)ethylenediamine dihydrochloride and 5% (v/v) H₂SO₄ in methanol, followed by 7 min of heating at 121°C. The amount of monosaccharides in EPS was analyzed by conversion of integrated density values (IDV) using the AlphaEaseFC 4.0 program (Alpha Inotech, USA).

Mushroom Tyrosinase Inhibition Assay of EPS from *F. castanea*

The inhibition activity of FEPS against mushroom tyrosinase was carried out as in our previous study [20]] with kojic acid as a positive control. Assays were completed as above with FEPS concentration from 0–20 mg/ml. The absorbance of the reaction was measured at 475 nm using a SpectraMax M3.

Cell Cytotoxicity Tests

SK-MEL-5 human melanoma cells and RAW264.7 mouse macrophage cells were obtained from the Korean Cell Line Bank (Korea) and maintained in RPMI 1640 and Dulbecco's modified Eagle's medium, fetal bovine serum purchased from GenDEPOT (USA), and penicillin and streptomycin purchased from Invitrogen

(USA) at 37°C in 5 % CO₂ [21, 22]. Cells grown in a 96-well plate at 2×10^4 cells/well were treated with various concentrations of FEPS (0–250 µg/ml) or β-arbutin (156.3–2,500 µM) at 37°C for 24 h. Then, 90 µl of culture medium was mixed with 10 µl of Ez-CyTox solution purchased from DOGEN (Korea). After 1 h at 37°C, plates were measured at 450 nm using a SpectraMax M3. Percent viability was determined as cell viability relative to the control.

Cellular Tyrosinase Activity Assay

SK-MEL-5 human melanoma cells grown in a 6-well plate at 2×10^5 cells/well were treated with different concentrations of FEPS (50–100 µg/ml) or β-arbutin (1–2 mM) and 500 nM alpha-melanocyte stimulating hormone (α-MSH) for 24 h. 150 µM kojic acid were used as a positive control. Then, the cells were harvested after trypsin treatment and washed twice with ice-cold PBS. The cell pellet was obtained by centrifugation at 12,600 ×g for 10 min and lysed in 50 mM PBS containing 1% Triton X-100 on ice for 30 min at 4°C. Lysates were cleared by centrifugation at 12,600 ×g for 20 min at 4°C. The protein concentration in the supernatant was determined using Bio-Rad DC protein assay, with bovine serum albumin as a standard. The reaction mixture consisted of cell-extracted protein (10 µg), 2.5 mM L-DOPA in 50 mM PBS buffer (pH 6.8) at 37°C for 1 h. The oxidation of L-DOPA was measured at 475 nm using a SpectraMax M3. Activity was calculated using the following formula: Tyrosinase activity (%) = (OD₄₇₅ of sample / OD₄₇₅ of control) × 100.

Measurement of Nitric Oxide Production

Nitric oxide production was determined as described previously [23]. RAW264.7 cells grown on a 96-well plate at 2×10^4 cells/well for 48 h were treated with FEPS at a concentration from 1.56–200 µg/ml with 1 µg LPS/ml for 24 h and 100 µM indomethacin was used as a positive control. Then, culture supernatant (80 µl) was mixed with Griess reagent (80 µl) for 20 min. The absorbance was measured at 540 nm using a SpectraMax M3. The amount of nitrite in the sample was calculated from a standard curve prepared with a sodium nitrite standard curve of (0–500) µM in the cell culture medium.

Zebrafish Experiments

Zebrafish eggs were obtained from the Zebrafish Center for Disease Modeling (ZCDM, Korea).

Zebrafish embryos were allocated in a 6-well plate, with 3 embryos/well of triplicate groups, containing 2 ml of embryo medium, which consisted of 5.03 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂·2 H₂O, 0.33 mM MgSO₄·2 H₂O, and 0.00001% (w/v) methylene blue. At 36 h post fertilization (hpf), FEPS of (100 and 400) µg/ml was treated to each well containing dechorionated zebrafish embryos, and phenotype-based evaluations of anti-melanogenic effects were performed. 1-phenyl-2-thiourea (PTU) and kojic acid (Sigma) were used as positive controls. At 54 hpf, embryos were transferred to the glass bottom for observation and

photography of the effect on the pigmentation of zebrafish under a Leica M205FA (Leica Microsystems, Germany) stereoscope in the National Instrumentation Center for Environment Management (NICEM, Seoul National University, Korea). The quantification of melanin pigmentation was conducted by measuring the estimated raw integrated density on zebrafish embryos using Image J software (National Institutes of Health, USA). The experimental protocols in this study were carried out with zebrafish larvae up to 54 hpf and therefore are not subject to the regulations of the Institutional Animal Care and Use Committee.

Statistical Analysis

Experiments were conducted in triplicate, and the data were shown as mean ± standard error of the mean (SEM). Statistical analysis was done using one-way ANOVA and Tukey *post hoc* multiple comparison tests on GraphPad Prism 8.0.2 (GraphPad Software, USA).

Results

Inhibitory Effect of Different Fungal Exopolysaccharides against Mushroom Tyrosinase

The crude EPS from 6 fungal species were examined for tyrosinase inhibitory activity. The crude EPS were extracted by ethanol and deproteinized by the Sevag method. The aqueous fractions were collected. The mushroom tyrosinase

Fig. 1. Inhibitory activity of the exopolysaccharides from different fungal EPS against mushroom tyrosinase.

EPS extracted from 1: *Abortiporus biennis*, 2: *Cryptoporus volvatus*, 3: *Fomitopsis castanea*, 4: *Phallus luteus*, 5: *Pholiota alnicola*, 6: *Pholiota limonella*. The inhibitory effect of exopolysaccharides against mushroom tyrosinase were carried out at reaction mixture containing 10 U/ml mushroom tyrosinase, 3.3 mM L-DOPA, and 10% of fungus exopolysaccharides in 50 mM potassium phosphate buffer (pH 6.8) for 10 min at 25°C.

inhibitory effects of EPS that was extracted from lyophilized culture supernatants from *Abortiporus biennis*, *Cryptoporus volvatus*, *Fomitopsis castanea*, *Phallus luteus*, *Pholiota alnicola*, and *Pholiota limonella* mycelia were shown in Fig. 1. Except for the EPS from *Phallus luteus*, the other EPS inhibited mushroom tyrosinase activity of 1–45% of original activity (Fig. 1). Among them, EPS from *Fomitopsis castanea* (FEPS) showed the strongest inhibitory effect against mushroom tyrosinase, and FEPS were selected for further biochemical studies.

Production of *Fomitopsis castanea* Exopolysaccharides Using Submerged Culture

The extraction yield of FEPS was 213.1 mg/l in DY media containing 2% (w/v) dextrose and 0.2% (w/v) of yeast extract. The concentration of protein in FEPS was 0.03%.

Monosaccharide Composition of *Fomitopsis castanea* Exopolysaccharides

After acid hydrolysis, monosaccharide composition was determined by TLC analysis (Fig. 2). Comparing the R_f value of standard compounds obtained from TLC analysis,

Fig. 2. Thin-layer chromatogram of monosaccharide composition of the EPS extract from *Fomitopsis castanea*.

Man: mannose; Glu: glucose; Ara: arabinose; Gal: galactose; Rham: rhamnose; S: acid hydrolysate of FEPS; M: standard mixtures (mannose, glucose, arabinose, galactose, rhamnose). FEPS (1 mg) were hydrolyzed by 0.5 M hydrochloric acid at 121°C for 30 min in a vial. The monosaccharide composition was analyzed using TLC with five ascents of acetonitrile/water (85:15, v/v).

FEPS from *Fomitopsis castanea* were composed of galactose (16.8%), glucose (34.5%), and mannose (25.8%) as predominant sugars (Table S1).

Mushroom Tyrosinase Inhibitory Effect

The details of the inhibitory effect against mushroom tyrosinase activity of FEPS of 0–20 mg/ml were studied (Fig. S1). FEPS at 10, 12, 14, 16, and 20 mg/ml inhibited this activity by 18.6, 27.4, 32.4, 43.8, and 72.4%, respectively. The IC_{50} of FEPS against mushroom tyrosinase was determined as 16.5 mg/ml. FEPS inhibited mushroom tyrosinase in a dose-dependent way.

Fig. 3. Cell viability (A), and cellular tyrosinase (B) on SK-MEL-5 human melanoma cells of EPS from *F. castanea* mycelial culture supernatants.

Cellular Tyrosinase Activity on SK-MEL-5 Human Melanoma Cells

Arbutin is currently used in the cosmetic industry as a skin brightening agent because it shows strong inhibition against tyrosinase from mushroom, mouse, and human [24, 25]. Thus, arbutin was selected as a positive control for this experiment. Cell viabilities of SK-MEL-5 human melanoma cells were shown in Fig. 3A. Since the cells exhibited a survival rate of over 95% at 250 $\mu\text{g/ml}$ (Fig. 3A), the cellular tyrosinase was conducted with concentration ranging (50–150) $\mu\text{g/ml}$. α -MSH was used to study the inhibitory effects of FEPS on the cellular tyrosinase expression level in SK-MEL-5 cells. The cellular tyrosinase activity of cells treated with α -MSH was assigned as 100%. Incubation for 24 h with FEPS of 50 or 100 $\mu\text{g/ml}$ or kojic acid of 150 μM resulted in tyrosinase inhibition of 63.9%, 83.3%, or 83.3%, respectively (Fig. 3B). β -arbutin did not show any cytotoxicity under assay condition (Fig. S2A). The inhibitory activity of 1–2 mM β -arbutin after 24 h incubation was 87.2% and 91.5%, respectively.

Inhibition of Nitric Oxide Production in LPS-Stimulated RAW 264.7 Cells

In this study, we investigated whether FEPS acted as an inhibitor or scavenger for nitric oxide (NO) and as an inflammatory mediator released from lipopolysaccharide-induced mouse macrophage cell model [26]. Production of NO was investigated after lipopolysaccharide (LPS) stimulation. Since the cells exhibited a survival rate of over 95% at 200 $\mu\text{g/ml}$ (Fig. 4A), the inhibition of NO production in LPS-stimulated RAW264.7 cells was conducted from 3.125–200 $\mu\text{g/ml}$ (Fig. 4B). LPS led to an increase in NO production when compared with the negative control, but FEPS at concentration of 3.125–200 $\mu\text{g/ml}$ caused a reduction in NO production in a dose-dependent manner when compared with the negative control. In detail, the inhibitory effect on producing NO in RAW264.7 treated with FEPS at concentrations of 3.125, 6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/ml}$ for 24 h were $12.5 \pm 0.8\%$, $13.8 \pm 2.1\%$, $21.3 \pm 3.8\%$, $40.1 \pm 1.5\%$, $53.9 \pm 0.2\%$, $63.6 \pm 0.8\%$, and $71.6 \pm 0.9\%$ as compared to the group treated with LPS only, respectively. The IC_{50} of FEPS against NO production from RAW264.7 macrophage cells was 42.8 ± 0.64 $\mu\text{g/ml}$ (Fig. 4B).

Evaluation of Melanogenic Inhibitory Effect Using the Zebrafish Model

Dechorionated zebrafish embryos at 36 hpf were incubated with FEPS at 100 and 400 $\mu\text{g/ml}$, and 10 mM kojic acid and 0.2 mM PTU as positive control (Fig. 5A). The FEPS at

Fig. 4. Cell viability (A), and nitric oxide production (B) on RAW264.7 cells of EPS from *F. castanea* mycelial culture supernatants.

400 $\mu\text{g/ml}$ significantly decreased the pigmentation of dechorionated zebrafish embryos (Fig. 5B). There are no reports on the antimelanogenesis effect of EPS on zebrafish embryos.

Discussion

Melanin plays an important role in the determination of eye, skin, hair color and human skin homeostasis, such as protection against ultraviolet irradiation, chemicals and scavenging toxic drugs [27]. However, abnormal accumulation of melanin in the skin as hyperpigmented spots affects its appearance. To treat skin and pigment abnormalities, many

Fig. 5. Effect of EPS from *F. castanea* mycelial culture supernatants on melanin synthesis in zebrafish (A) and the quantification of melanin pigmentation was conducted using Image J software (B).

Dechorionated zebrafish embryos were treated from (36–54) hpf with 10 mM kojic acid, 0.2 mM PTU, and 100 and 400 µg/ml FEPS, and the effects on pigmentation were assessed using a stereomicroscope. (1; control, 2; 0.2 mM PTU, 3; 400 µg/ml FEPS, 4; 100 µg/ml FEPS, 5; 10 mM kojic acid) (B) * $p < 0.1$, ** $p < 0.05$ was considered as statistically significant compared with the control.

brightening agents have been reported and applied in cosmeceutical, food, and pharmaceutical industries. The natural inhibitors against tyrosinase have increased because safety is strictly monitored in the food and cosmetic industries. In this study, we examined antimelanogenesis of 6 different fungal exopolysaccharides. Among them, EPS from *Fomitopsis castanea* was selected due to its high tyrosinase inhibitory activity. According to our best knowledge, this is the first study demonstrating anti-melanogenesis of exopolysaccharides from *Fomitopsis castanea*. The type of strain, medium components, and physical conditions maintained during fermentation have effects on the amount of polysaccharide production, the

composition of final products, structure, molecular weight, and functional properties of EPS [9, 28, 29]. The protein concentration in FEPS is lower than that in FEPS extracted by Guo and Chi [17]. The predominant sugars of FEPS in this study were galactose (16.8%), glucose (34.5%), and mannose (25.8%) while Guo and Chi reported that EPS from *Fomitopsis castanea* mainly consists of rhamnose (43.4%), arabinose (0.09%), mannose (0.13%), glucose (51.32%), and galactose (5.06%) [17].

There are various factors that affected the monosaccharide content in exopolysaccharides such as monosaccharide composition [30, 31], metal ions [32], aeration rate, agitation speed, pH, temperature [33, 34]. Even with the same strain, the chemical composition and quality of EPS are also influenced by conditions such as nutrient status and growth phase [35]. In some cases, one strain can produce different exopolysaccharides with different molecular weight; *Bacillus thermoantarcticus* synthesized two different EPSs, one of these was mainly composed of mannose and glucose in a ratio of 1:0.7, and the other was a mannan [36]. In this study, we used DY media containing 2% (w/v) dextrose and 0.2% (w/v) of yeast extracted, while Guo and Chi used 1% (w/v) peptone, 1% (w/v) beef extract, 5% (w/v) yeast extract, 2% (w/v) glucose, 0.1% (v/v) Tween-80, 0.5% (w/v) sodium acetate, 0.2% (w/v) diammonium citrate, 0.2% (w/v) dipotassium phosphate, 0.058% (w/v) of magnesium sulfate, and 0.025% (w/v) of manganese sulfate [17]. In addition, we used crude exopolysaccharides to analyze monosaccharide content instead of purified EPS as reported by Guo and Chi [17]. Therefore, the different monosaccharide content of EPS from *Fomitopsis castanea* between our finding and the previous report [17] was possibly the result of various culture conditions.

Mushroom tyrosinase and α -MSH-stimulated SK-MEL-5 human melanoma cells are used extensively for anti-melanogenesis studies of FEPS. In melanocytes, FEPS inhibited cellular tyrosinase in a dose-dependent manner (Fig. 3B). Keratinocytes and other cells around melanocytes release several melanogenic factors, such as a nitric oxide (NO) in response to proinflammatory cytokines [37]. In human melanocytes, NO donors have been reported to increase tyrosinase activity and melanin synthesis [37]. The cyclic guanosine 3'-5'-monophosphate (cGMP) pathway is reported to be a major mechanism for NO-induced melanogenesis for the up-regulation of tyrosinase gene expression [38]. NO was demonstrated as a paracrine mediator of UV-induced melanogenesis [37]. Although NO has been reported to play a beneficial role in the physiological

process, such as the regulation of neuronal communication [39], they can also react with oxygen, produce reactive molecules, and aggressively attack biomolecules, resulting in inflammation [39, 40]. We found that FEPS inhibit the making of NO in LPS-stimulated RAW264.7 macrophages cells. However, further studies are needed to evaluate the activation of the intracellular signaling pathways of FEPS. We also examined the antimelanogenesis effect of FEPS through an in vivo test using zebrafish embryos. The zebrafish embryo is a general vertebrate model system for biochemical research with high physiological and genetic correspondence to mammals in terms of melanocytes and melanosomes. In addition, they are also small in size, easy to handle, have many offspring each generation, and absorb drugs efficiently through their skin and gills [41, 42]. Therefore, zebrafish embryos can replace animal subjects in animal experiments [41–43]. Melanin pigments can be detected on the zebrafish surface, allowing simple observation of the pigmentation process [44]. Amounts of 100 and 400 µg/ml of FEPS were added to each well containing dechorionated zebrafish embryos and at 400 µg/ml of FEPS decreased significantly the pigmentation of the embryos. FEPS could effectively reduce melanogenesis via suppression of tyrosinase activity in zebrafish.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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