Inhibitory Effects of Mung Bean (*Vigna radiata* L.) Seed and Sprout Extracts on Melanogenesis

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Abstract Inhibitory effect against melanogenesis and the *in vitro* tyrosinase inhibitory activity of ethyl acetate (EtOAc) and methylene chloride fractions of 80% methanol extracts of mung bean (*Vigna radiata* L.) seeds and sprouts were determined. EtOAc extract fractions from mung bean sprouts germinated for 12 h (33.5%), 1 day (56.5%), and 2 days (47.9%) inhibited melanogenesis more effectively than arbutin (16.3%). The *in vitro* tyrosinase inhibitory activity was higher in an EtOAc extract fraction from mung bean sprouts germinated for 2 days (70.5%). EtOAc extract fractions from mung bean sprouts germinated for 1 day showed excellent whitening effects due to the flavone vitexin. Extracts from mung bean sprouts germinated for 1 day can be used as a novel whitening cosmeceutical ingredient.

Keywords: mung bean, sprout, vitexin, isovitexin, whitening effect

Introduction

Melanocytes are responsible for synthesis of melanin in melanosomes (organelles), which were transported to keratinocyte for protection of its nucleus from ultraviolet rays. Melanin is a biopolymer material that is present in plant and animal systems and is a key factor for determination of the color of human skin. Melanin is biosynthesized by melanin-producing melanocytes present in the basal layer of the skin and is transported by keratinocytes through dendrites, moving to the outer layer of the skin during the process of keratinization (1-3). Tyrosinase is a speed-adjustment factor for catalysis of the initial step in melanin synthesis, and the 2 tyrosinase-related proteins TRP-1 and TRP-2 regulate melanin synthesis via L-tyrosine conversion to 3,4-dihydroxyphenylalanine (DOPA) and DOPA quinone. After a further series of conversions, a mixture of dark brown melanin known as eumelanin, and reddish yellow melanin known as pheomelanin, is produced in melanosomes (1,4,5). Synthesized melanin serves a function of skin protection via suppression of aging and actinic keratosis caused by UV light (6-11). However, if excessive melanin is produced, or if the function of skin cells is reduced via aging, melanin can be deposited on the surface of the skin, appearing as spots and freckles that are induced by toxicity caused by melanin precursors, which can also induce cell death (12-14). Therefore, development of a new whitening agent would be helpful for maintenance of bright and healthy skin (15-17).

The mung bean (*Vigna radiata* L.) is an herbaceous plant. One year old beans from India, also referred to as nok du (nok du), an du (安豆),

or gil du (吉豆) (18) were used in this study. Mung beans have been used for many years in oriental medicine for detoxification, as a treatment for erysipelas and diarrhea, for alleviation of heat stroke, and for reduction of swelling in summer (19). In addition, mung bean seeds and sprouts are widely used in China, India, Bangladesh, and Southeast Asia (20-21) and have been reported to exert antimicrobial, anti-inflammatory (22-27), and anti-melanocytic effects (28), antitumor (29), anti-oxidant (30), antidiabetic effect (31). After germination, mung beans exhibit stronger biological activities and more secondary metabolites because relevant biosynthetic enzymes are activated during the initial stages of germination. Thus, germination is thought to improve the nutritional and medicinal qualities of mung beans (22,27).

In this study, extracts of mung bean seed and its sprouts were investigated for inhibitory effects against melanogenesis using mushroom tyrosinase and B16F1 melanoma cells. In addition, HPLC analysis was used for confirmation of changes in main component contents of mung bean seed and germinated sprout extracts for inhibition of melanogenesis. Thus, an optimal germination time was determined and confirmed to be important when using extracts of germinated mung bean seeds as a functional cosmetic whitening material.

Materials and Methods

Chemicals and materials Disodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$),

sodium phosphate monobasic dehydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), sodium chloride (NaCl), and the solvents ethanol, methanol, ethyl acetate, methylene chloride, and n-hexane of analytical grade were used. Dulbecco's Modified Eagle's Medium (DMEM) (PAA Co., Pasching, Austria), fetal bovine serum (FBS) (PAA Co.), penicillin-streptomycin, trypsin (PAA Co.), and bovine serum albumin (BSA) (PAA Co.) were used for cell cultures. HPLC grade acetonitrile was purchased from J. T. Baker (Phillipsburg, NJ, USA). Standard formulations of L-tyrosine, *N*-succinyl-(Ala)₃-*p*-nitroanilide, tyrosinase (11.9 mg of solid, 4,276 units/mg of solid), arbutin, vitexin, and isovitexin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Organic mung bean seeds were purchased in January, 2014 from Jayeonmidam (Gwangju, Korea).

Germination conditions Mung bean seeds were washed thoroughly under running tap water, then soaked in tap water for 1 h at room temperature of 20°C. After removal of excess water, soaked mung bean seeds were placed into a sprouter (260x160 mm) at 25 g of seed/cup and kept in the dark at room temperature for germination. Resultant sprouts were rinsed with deionized water every 12 h with exposure to light for 10 min for a total germination period of 7 days. Resultants sprouts were taken after 0 h (no soaking, seed only), 12 h, and 1, 2, 4, and 6 days. Resultants sprouts were immediately freeze-dried (lyophilizer; Ilshinbiobase, Seoul, Korea). Freeze-dried resultants sprouts were stored at -78°C and 5 mm Torr by using a lyophilizer prior to analysis.

Preparation of mung bean seed and sprout extracts Freeze-dried germinated mung bean sprouts (100 g) were crushed in a blender, and then ground resultants sprouts were subjected to extraction using 1.5 L of 80% methanol for 1 day at room temperature. After 80% methanol sprout extracts were concentrated in a rotary evaporator (BUCHI, Meierseggrasse, Switzerland), resultant concentrated extracts (the concentrated form of the methylene chloride fraction of mung beans) were treated 3 times with methylene chloride, then the fractions were concentrated in a rotary evaporator for solvent removal, then used for experimentation. The aqueous fraction of 80% methanol extracts was treated 3 times with ethyl acetate, which separated out as a fraction that was then concentrated under use of a rotary evaporator to remove the organic solvent (the concentrated form the ethyl acetate fraction of mung beans). Each fraction was freeze-dried and stored by using a lyophilizer, prior to subsequent experimental use.

Inhibitory effect of mung bean sprouts on melanogenesis with sprouting time

Cell culture: The mouse melanoma cell line B16F1 was provided by the Kyunghee University Skin Biotechnology Center (Yongin, Korea). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2 .

Cell viability: Cell viability was determined using an 3-(4,5-

dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT, Sigma Chemical Co.) assay. B16F1 melanoma cells were seeded on a 96 well plate and incubated at 37°C in 5% CO_2 for 24 h. The methylene chloride and ethyl acetate fractions obtained using different germination conditions for mung bean sprouts were added at concentrations from 10-250 $\mu\text{g/mL}$. After 72 min, an MTT solution at 2 $\mu\text{g/mL}$ was added to each well, and the cells treated with different fraction of different germination conditions were incubated for 3 h. Resulting formazan crystals were dissolved in dimethyl sulfoxide and quantified based on measurement of optical density values at 570 nm using a microplate reader (ELISA reader, Tecan Schweiz AG, Männedorf, Switzerland). Mung bean sprout fractions obtained at different germination times were determined the optimal ratio of concentration of 10 $\mu\text{g/mL}$ based on cell viability testing and carried out other experiment (data not shown).

Melanin content assay: The melanin content was determined using the method described by Hosoi (32). B16F1 melanoma cells were seeded and cultured on a 6-well plate at a density of 1×10^5 cells/well then incubated at 37°C in 5% CO_2 with CO_2 incubator (MCO-19AIC; Sanyo, Japan). After 24 h, cell adherence was confirmed, and the culture medium was replaced with fresh medium after the cells had been washed with PBS, after which cells were treated with 200 nM α -melanocyte-stimulating hormone (α -MSH), methylene chloride, and 10 $\mu\text{g/mL}$ ethyl acetate fractions for different germination conditions of mung bean sprouts. After 72 h, cells were harvested, and the supernatant was removed. The cell pellet was solubilized at tube in 1 N NaOH containing 10% DMSO at 80°C for 3 h. The melanin content was quantified based on measurement of optical density values of pellets at 405 nm using a micro plate reader.

Determination of cell lysates: B16F1 melanoma cells were seeded on a 6 well plate at a density of 10^5 cells/well and incubated at 37°C in 5% CO_2 incubator. After 24 h, cell adherence was confirmed, the culture medium was replaced with fresh medium, and cells were treated for 72 h with 200 nM α -MSH and 6.25, 12.5, 25, and 50 μM concentrations of vitexin and isovitexin, which did not exert an influence on cell viability (data not shown). After 72 h, cells were harvested, and the supernatant was removed. The cell pellet was solubilized at tube in 1 N NaOH containing 10% DMSO, and the melanin concentration was observed using the unaided eye.

In vitro inhibition of tyrosinase: Tyrosinase is an enzyme that determines the overall reaction rate of the 3,4-dihydroxy-L-phenylalanine (DOPA) oxidation process in which L-tyrosine converts DOPA-quinone in the melanogenesis pathway. Thus, inhibition of tyrosinase is useful for determination of a whitening effect. Thirty four μL of L-tyrosine at 0.3 mg/mL, 60 μL of 0.1 M potassium phosphate buffer (pH 6.8), and 3 μL of mung bean sprout extracts were mixed and incubated at 37°C for 10 min in incubator. After 10 min, the mixed compound were placed in an ice water bath to stop the reaction. The mixed compound were quantified by measuring their optical density values at 570 nm using a microplate reader.

$$\text{Inhibitory effect (\%)} = \left\{ 1 - \left(\frac{\text{Experiment} - \text{Blank}}{\text{Control}} \right) \right\} \times 100(\%)$$

Comparison of flavonoid contents and analysis of mung bean sprout extracts After methylene chloride and ethyl acetate fractions of mung bean sprout extracts were dissolved in 100% EtOH, resultant extract solutions were filtered through 0.45 μm syringe filters (Merck Millipore, Billerica, MA, USA), and solutions were then subjected to TLC and HPLC analysis. Subsequent HPLC analysis used an LC-20AT (Shimadzu, Kyoto, Japan), a UVD 170s Dionex UV-Vis detector (Shimadzu), and a Shim-pack (VP-ODS, L: 250 mm, LD: 4.6 mm) C18 analytical column (5 μm , 25094.6 mm). Ingredient confirmation was performed using TLC with previously reported spectroscopic data, flavonoid R_f values, and band colors obtained using the ultraviolet chromogenic method. The HPLC mobile phase was a mixture of 2% (v/v) acetic acid in distilled water and 0.5% (v/v) acetic acid in 50% (v/v) acetonitrile. The detection wavelength was set at 370 nm and the flow rate was 1.0 mL/min. Analytical conditions were 0-45 min at 25% solvent B, 45-55 min at 25-100% solvent B, and 55-60 min at 100-30% solvent B.

Analysis of flavonoid components using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) A Thermo-Finnigan Surveyor instrument (Thermo Scientific, Waltham, MA, USA) equipped with an auto sampler (Thermo Scientific) and a PDA-UV detector (Thermo Scientific) was used. Mass spectrometric analyses were performed using a Thermo-Finnigan LCQ Deca XP plus ion trap mass spectrometer (Thermo Scientific) with an ESI interface (Thermo Scientific). Phenomenex columns (P/No. 00D-4424-Y0, Desc. Synergi 4u Fusion-RP 80A, size 100x3.00 mm, 4 micron, S/No. 460415-1) were used for chromatographic separations. The injection volume was 5 μL and the flow rate was 200 $\mu\text{L}/\text{min}$, Desc. Synergi 4u Fusion-RP 80A, size 0.1% formic acid (solution A) and acetonitrile (solution B). Gradient elution occurred at a flow rate of 200 $\mu\text{L}/\text{min}$. The total run time was 30 min and ionization of analytes was carried out using electrospray ionization (ESI). The capillary temperature was maintained at 275°C ionization of analytes was carried out kV. and the sheath and Aux gas was set at 30 and 5 units, respectively. The capillary voltage was set at 45 V in the positive mode and -15 V in the negative ionization mode. The average scan time was 0.01 min and the average time to change polarity was 0.02 min. The collision energy (CE) was generally chosen to maintain an approximate 35% abundance of the precursor ion.

Statistical analysis Statistical evaluation of data was performed using a one-way analysis of variance (ANOVA). The Bonferroni multiple comparison test was used for comparison of significance of differences between groups at $p < 0.05$ and $p < 0.01$. Data were expressed as a mean value $\pm$ standard deviation (mean $\pm$ SD) ($n = 3$).

Fig. 1. Melanogenesis inhibitory effects of ethyl acetate (EtOAc) and methylene chloride (MC) fractions of mung bean (*Vigna radiata* L.) seeds (time=0) and sprout extracts (12 h, 1, 2, 4, and 6 days) in B16F1 cells with 200 nM α -MSH. Values are reported as mean $\pm$ standard error of the mean * $p < 0.05$, ** $p < 0.01$ compared with controls.

Results and Discussion

Melanin synthesis inhibitory activity The α -melanocyte-stimulating hormone (α -MSH) induces melanogenesis. B16F1 melanoma cells were treated with 200 nM α -MSH and ethyl acetate and methylene chloride extract fractions from mung bean seeds (time=0) were evaluated for inhibitory actions and sprouts germinated for 12 h, 1, 2, 4, and 6 days (Fig. 1). Melanin contents were 148.6% at 0 h, 102.6% at 12 h, 79.6% at 1 day, 88.2% at 2 days, 123.9% at 4 days, 135.6% at 6 days, and 136.1% for controls (α -MSH). Inhibitory effects against melanogenesis were especially apparent for ethyl acetate fractions of mung bean sprouts with values of 33.5% for 12 h of germination, 56.5% for 1 day, and 47.9% for 2 days. Inhibitory effects against melanogenesis were weakly indicated in methyl chloride fractions with values of 134.9% for germination time=0, 127.3% for 12 h, 146.1% for 1 day, 133.0% for 2 days, 134.7% for 4 days, and 134.5% for 6 days. Melanogenesis inhibition effects of ethyl acetate fractions at 12 h, 1 day, and 2 days were greater than for arbutin at 16.3%.

In vitro tyrosinase inhibitory activity Tyrosinase acts as a catalyst for conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (DOPA), and participates in oxidation of DOPA quinone from DOPA in biosynthesis of melanin. The follow-up reaction after the 2 oxidation reactions occurs spontaneously. Therefore, the reaction catalyzed by tyrosinase determines the overall reaction rate, and inhibition of tyrosinase activity is an important factor for evaluation of depigmentation effects (33). Inhibitory activities of mung bean seed and sprout extract fractions on *in vitro* tyrosinase activity are shown in Fig. 2. *In vitro* tyrosinase inhibitory activities of each fraction evaluated at the same concentration of 1,000 $\mu\text{g}/\text{mL}$, which was used as the effective concentration of the extract, showed different tendencies for

Fig. 2. *In vitro* inhibition of mushroom tyrosinase by ethyl acetate (EtOAc) (A) and methylene chloride (MC) fractions (B) of mung bean (*Vigna radiata* L.) seeds (0 h) and sprout extracts (12 h, 1, 2, 4, and 6 days). Values are reported as mean $\pm$ standard error of the mean * p <0.05, ** p <0.01 compared with controls.

inhibition of B16F1 melanoma cells during melanogenesis. Inhibition values for tyrosinase activity by the ethyl acetate extract fraction of mung bean sprouts based on germination time were 24.0% at 0 h, 31.5% after 12 h, 35.9% after 1 day, 70.5% after 2 days, 38.7% after 4 days, and 30.2% after 6 days. After 2 days of germination time, the ethyl acetate fraction of mung bean sprout extracts showed inhibition of tyrosinase activity. Furthermore, inhibition of tyrosinase activity by the methylene chloride fraction of mung bean sprout extracts was 31.4% at 0 h, 34.6% after 12 h, 26.1% after 1 day, 31.4% after 2 days, 27.7% after 4 days, and 24.6% after 6 days. The methylene chloride fraction of extracts from mung bean sprouts germinated for 12 h showed only slight inhibition of tyrosinase activity. The evaluated concentration (1,000 μ g/mL) of the fraction was higher than the concentration (10 μ g/mL) used for melanogenesis inhibition. However, this was not considered to be important.

Composition and content analysis of mung bean seed and sprout extracts

TLC analysis: A TLC chromatogram of the ethyl acetate extract fraction of mung bean sprouts germinated for 1 day is shown in Fig. 3. The highest inhibitory level against melanin synthesis in cells was ethyl acetate extract fraction of mung bean sprouts germinated for 1 day (79.6%). The standard materials vitexin and isovitexin, the main components of the ethyl acetate fraction of mung beans, were identified using TLC to confirm the presence of these components.

Fig. 3. Thin layer chromatography (TLC) chromatogram of the ethyl acetate fraction from mung bean extracts and reference. The eluent system used was ethyl acetate:chloroform:formic acid:water=8:1:1:1 (v/v). 1, isovitexin standard; 2, ethyl acetate fraction from mung bean extracts; 3, vitexin standard.

The ethyl acetate extract fraction from mung bean sprouts germinated for 2 days separated into 8 bands under mobile phase conditions for separation of flavonoid glycosides. Respective bands were identified as VRS-1 to VRS-8 from the bottom of the chromatogram. The 2 bands that are clearly separated in Fig. 3 were used for further experimentation. These 2 bands were identified using reference materials for comparison. VRS-1 with an R_f value=0.36 was identified as isovitexin, and VRS-2 with an R_f =0.51 was vitexin.

HPLC analysis: HPLC analysis was performed to determine the presence of vitexin and isovitexin in the ethyl acetate fraction of extracts from the mung bean sprouts germinated for 1 day. Peaks for vitexin at 37.063 min and isovitexin at 41.241 min were confirmed (Fig. 4). In addition, the HPLC chromatogram of the ethyl acetate fraction of extracts from mung bean sprouts germinated for 1 day showed a superior whitening effect (Fig. 4C). Reference materials were used to demonstrate that peaks 1 and 2 were co-migrated with vitexin and isovitexin, respectively, and were confirmed to have the highest intensity values, all consistent with TLC results.

LC/ESI-MS/MS analysis: LC/ESI-MS/MS was used for structural analysis of VRS-1 (Table 1). A molecular ion $[M-H]^-$ was identified at m/z =431.1 for VRS-1 and m/z =431.1 for VRS-2) in negative ion mode (data not shown). Therefore, VRS-1 and VRS-2 of mung bean sprout extracts were identified as apigenin-6-C-glucoside (isovitexin) and apigenin-8-C-glucoside (vitexin), respectively.

Content analysis: Identities of main components were confirmed along with content changes based on germination time using HPLC. Vitexin contents were 23.0, 36.7, 37.9, 20.5, 3.7, and 2.9%, and isovitexin contents were 4.1, 7.5, 8.5, 2.9, 1.6, and 1.4% at 0, 0.5, 1, 2, 4, and 6 days, respectively. The ethyl acetate fraction of mung bean sprout extracts germinated for 2 days, which showed a superior whitening activity in analysis of intracellular inhibition of melanin biosynthesis, contained high concentrations of vitexin and isovitexin (Table 2). Furthermore, concentrations of the main extract components from germinated mung bean sprouts were higher than concentrations in extracts from non-germinated mung bean seeds. In particular,

Fig. 4. HPLC profile of vitexin (A), isovitexin (B) and the ethyl acetate fraction from mung bean sprout extracts (1 day) (C) at λ (c) at 335 nm.

vitexin and isovitexin contents showed increases after 12 h and 1 day. However, at 2, 4, and 6 days, vitexin and isovitexin contents showed decreases. During initial sprouting, mung bean shells were observed during growth. However, the mung bean hull peeled away from the sprout during sprout germination. In this process, the contents of the main vitexin and isovitexin components were probably affected. Similar effects were observed during melanogenesis in B16F1 melanoma cells. However, as the germination time increased from 4 to 6 days, vitexin and isovitexin contents decreased, and the whitening activity was also reduced.

Inhibitory effects of vitexin and isovitexin on melanogenesis The inhibitory effect of melanogenesis with 200 nM α -MSH on B16F1 cells was measured based on analysis of melanin contents using a UV spectrophotometer at $\lambda_{\text{max}}=405$ nm. Dose-dependent inhibitory effects against melanogenesis were exerted by vitexin and isovitexin. Melanin contents were quantified for the α -MSH control of 150.4%, with vitexin values of 150.5, 151.5, 139.8, and 119.6% and isovitexin values of 147.7, 134.8, 143.0, and 136.4% at 6.25, 12.5, 25.0, and 50.0 μM , respectively (Fig. 5A and 5B). In particular, the melanogenesis inhibitory effects of vitexin compared with the positive α -MSH

Table 1. Structural analysis of VRS-1 and VRS-2 followed by LC/ESI-MS/MS

Compound	Molecular cut
VRS-1	R.T 17.8 min; UV, 239, 269, 335 nm; MS, 431.1 [M-H] ⁻ ; MS/MS [M-H] ⁻ , 413.1 [(M-H)-18] ⁻ , 341.2 [(M-H)-90] ⁻ , 311.2 [(M-H)-120] ⁻
VRS-2	R.T 16.2 min; UV, 239, 268, 335 nm; MS, 431.1 [M-H] ⁻ ; MS/MS [M-H] ⁻ , 341.1 [(M-H)-90] ⁻ , 311.1 [(M-H)-120] ⁻

Table 2. Contents of vitexin and isovitexin with germination time

Germination (days)	Vitexin ($\mu\text{g/g}$) ¹⁾	Isovitexin ($\mu\text{g/g}$) ¹⁾
0	6.90	1.22
0.5	11.02	2.24
1	11.37	2.55
2	6.16	0.88
4	1.13	0.47
6	0.89	0.43

¹⁾Contents of vitexin and isovitexin/freeze-dried mung bean and/or mung bean sprouts

control were 21% and 61.1% at 25 and 50 μM , respectively. Thus, isovitexin exhibited a lower degree of whitening activity than vitexin. Therefore, the ethyl acetate fraction of mung bean seed extracts for germination of 1 day showed inhibitory effects against melanogenesis due to vitexin.

Visual confirmation of B16F1 melanoma cell lysates with vitexin and isovitexin Visual confirmation of B16F1 melanoma cells lysates treated with vitexin and isovitexin, the active compounds in the depigmentation process, was performed. Cells treated with 200 nM α -MSH showed increased melanin contents and a dark brown color. However, melanin contents decreased in a dose-dependent manner with vitexin and isovitexin treatments, in contrast to treatment with α -MSH alone (Fig. 5C and 5D). Vitexin markedly decreased the melanin content to 50 μM . Thus, vitexin exhibited strong inhibitory effects against melanogenesis.

In conclusion, the whitening effects of the ethyl acetate and methylene chloride fractions of mung bean seed extracts and extracts of sprouts germinated for 0 h, 12 h, 1, 2, 4, and 6 days were determined. The main components of the ethyl acetate fraction of mung bean sprout extracts with germination for 1 day, which exhibited the strongest whitening effects, were vitexin at 37.9% and isovitexin at 8.5%. Vitexin and isovitexin both exerted a high degree of whitening effect in tyrosinase and melanin biosynthesis inhibition assays. The mechanism of the inhibitory effect on melanogenesis based on germination time of mung bean sprouts used in extracts should be determined and additional studies are required to evaluate the site-specific activity of mung bean sprout extracts. This study confirmed that mung bean sprout extracts exerted whitening activities and that vitexin was the main component involved in this activity. In order to increase the whitening activity of mung bean sprout extracts, germination time and the extraction solvent are important. The whitening activity based on sprout germination time was confirmed to be closely related to changes in main component contents.

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Disclosure The authors declare no conflict of interest.

Fig. 5. Inhibition of melanogenesis by vitexin (A), isovitexin (B) in B16F1 melanoma cells with 200 nM α -MSH, and photographs of lysates of B16F1 melanoma cells treated with vitexin (C) and isovitexin (D) in the presence of 200 nM α -MSH for 72 h. 10,000 cells were collected and dissolved with 1N NaOH containing 10% DMSO. Values are reported as mean $\pm$ standard error of the mean ($n=3$). * $p<0.05$, ** $p<0.01$ compared with controls.

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