The potential neuritogenic activity of aqueous extracts from *Morchella importuna* in rat pheochromocytoma cells

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Abstract The aim of this study was to explore the neuritogenic effects of aqueous extracts from the fruiting bodies of *Morchella importuna* (MEA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was carried out to assess the cytotoxicity of MEA. Neurite outgrowth stimulation assay was used to evaluate the potentiation of neuritogenic activity induced by MEA. The specific inhibitors for TrkA, MEK/ERK and PI3K signaling pathway were served to clarify the mechanism of MEA's neuritogenic effects. It was shown that MEA could mimic neuritogenic activity of NGF, a kind of representative neurotrophic factors with no significant cytotoxicity, and stimulate neurite outgrowth in a dose-dependent manner of PC12 cells. The neuritogenic activity induced by MEA required activity of PI3K/Akt and MEK/ERK1/2 signaling pathways, as well as parts of TrkA receptor. Accordingly, MEA could be used as a promising neuritogenic-stimulation compound for nervous diseases treatment.

Keywords *Morchella importuna* · Neuritogenic effect · Neurodegenerative disease · Nerve growth factor · Culinary-medicinal mushroom

Introduction

Senescence of neurons was regarded as one of the main causes of age-dependent neurodegenerative diseases such as Parkinson and Alzheimer's disease [1]. Nerve growth factor (NGF), one of the most representative neurotrophic factors (neurotrophins) which played distinctive roles in promoting neuronal survival, proliferation and development, had dual biological functions of neuronal nutrition and neuritogenic activity [2] and even been regarded as a potential therapeutic agent for treating neurodegenerative disorders [3]. It has been reported that the decrease of NGF in mouse's brain can lead to neurodegeneration and Alzheimer's-like symptoms. Furthermore, the learning ability and memory were improved in aged anti-nerve growth factor transgenic mouse after administration of NGF [4]. Thus, NGF was identified as a potential therapeutic agent to treat the disorder of neurodegeneration. However, NGF-intended treatment had been restricted by some difficult challenges. First, NGF was unstable and unable to cross the blood–brain barrier for large molecular polypeptide structure [5]. Second, sufficient quantities of NGF must be delivered so as to stimulate neurite outgrowth [6]. To address these shortcomings, some alternative strategies must be explored to overcome the shortages of NGF.

Mushrooms can not only be used as food, but can also be used to treat different diseases for thousands of years all over the world. Thus, culinary-medicinal mushrooms are more than just vegetables, they can be considered as functional foods [7]. In traditional western medicines,

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extracts of medicinal mushrooms have long been well known as one of the most fascinating parts [8]. Modern medical researches demonstrated that mushrooms have the function of anti-tumor, anti-cancer, anti-oxidants, antiviral, anti-microbial, anti-inflammatory, anti-diabetic and immune modulating activities [9]. Moreover, available evidence suggested that mushrooms possess neuritogenic effects [10]. The hot aqueous as well as ethanolic extracts, even crude polysaccharides from *Lignosus rhinocerotis* (Cooke) Ryvarden (tiger's milk mushroom) could stimulate neuritogenesis in the PC12 cells [11]. They may contain neuroactive compound(s) that mimic the neuritogenic activity of NGF. *Ganoderma lucidum* (Leyss. ex Fr.) Karst., *Hericium erinaceus* (Bull. Fr.) Pers. as well as *Sarcodon scabrosus* (Fr.) P. Karst., also had remarkable neuritogenic activities [12, 13].

Morchella spp. ascocarp (morel) was one of the world's most sought-after mushrooms with a unique appearance and delicate taste, also had been appreciated for its nutritional and medicinal properties [14]. Spectacularly, it was used in Traditional Chinese Medicine (TCM) to treat indigestion, excessive phlegm and shortness of breath for hundreds years [15]. Recently, it was found that morels had a significant antitumor activity for they could induce apoptosis of Hela cells and HepG2 cells through arresting cell cycle progression at the G0/G1 phase [16, 17]. Like many other edible fungi, strong antioxidant activity was found in morels. The extra-polysaccharides as well as crude polysaccharides isolated from morels had a remarkable effect on alleviating oxidative stress [18, 19]. Moreover, with cholesterol-lowering ability, morels protect against cardiovascular diseases [20]. In addition, it has more functions, like more exhibited hepatoprotective activity, resisting gentamicin and cisplatin induced nephrotoxicity by restoring the depleted antioxidant defense system [21, 22].

Previously, the multi beneficial effects about morels were mainly focused on its mycelia and ferment products, largely due to the lack of fruiting bodies. Currently, successful cultivation made the fruiting body of morels become a potent candidate for the medicinal development. However, rather limited information was available about the neuronal activity of morels, let alone the detailed mechanisms on how to induce neuritogenesis. In this work, as the first step to examine potential neural action(s) of morels, the cultured rat pheochromocytoma PC12 cells have been widely used as an in test tube model system to investigate different neuronal as the PC12 cell line can respond to NGF with a big alter in phenotype and also can prolong life and extense the neurite [23]. The potentiation of neuritogenic activity of aqueous extracts from morels was evaluated. Moreover, the signaling pathways involved

in neuronal differentiation induced by morels were investigated by specific inhibitors.

Materials and methods

Materials and chemicals

PC12 cells (ATCC CRL-1721) were kindly provided by Dr. Mengyao Yu from Sichuan Academy of Traditional Chinese Medicine. The reagents for cell culture, including F-12 K medium, horse serum (HS), fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were got from Gibco (Grand Island, NY, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, NGF-7S from murine submaxillary gland, phosphate buffered saline (PBS), dimethylsulfoxide (DMSO), anti-neurofilament 200 (anti-NF-200), 5-diphenyltetrazolium bromide (MTT) and antibodies that found in rabbit were got from Sigma (Louis, MO, USA). Antibodies for TrkA, ERK, Akt and corresponding phosphorylated antibodies were from Cell Signaling Technology (Cell Signaling Technology, USA). ProLong[®] Gold Antifade Reagent with DAPI (4-6-Diamidino-2-phenylindole) were bought from Life Technologies Corporation (California, USA) and also anti-rabbit IgG Fluorescein, isothiocyanate (FITC) antibody that stimulated in goat was obtained from Santa Cruz Biotechnology (Dallas, TX, USA); GADPH was bought from Cell Signaling Technology (Cell Signaling Technology, USA). MEK inhibitor (U0126, PD98059), TrkA inhibitor (GNF5837) and PI3K inhibitor (LY294002) were obtained from Selleckchem (Houston, TX, USA). The other reagents and chemicals were of analytical grade.

Preparation of aqueous extracts

The fruiting bodies of *Morchella importuna* Kuo et al. were collected from the experimental field of Sichuan Academy

Fig. 1 *Morchella importuna* collected from the experimental field of Sichuan Academy of Agricultural Sciences

of Agricultural Sciences (SAAS) (Fig. 1), a voucher's specimen had been preserved in the Biotechnology and Nuclear Technology Research Institute of Sichuan Academy of Agricultural Sciences, voucher Cyl158. Briefly, the fruiting bodies of *M. importuna* were shade dried at the temperature of 37 °C in AC fitted room with dehumidifiers. 200 g fruiting bodies of *M. importuna* were soaked with 95% EtOH for 24 h. The residue was dried and then immersed at a percentage of 1:20 (w/v) distilled water and agitated on a shaker 150 rpm at the indoor temperature all over the night. And then the compound was boiled in a water bath for 6 h followed by the sediment being discarded. The resulting water extraction of morels (MEA) were freeze-dried with 23.6% (w/w) of yield and then kept at − 20 °C before use.

Cell viability and cytotoxicity assay

PC 12 cells shall be planted in the environment of F-12 K medium which include 2.5% FBS, 15% HS, 100 µg/mL of streptomycin, and 100 U/mL of penicillin in a wet climate with 5% CO₂ at the temperature of 37 °C. The mitochondrial-dependent reduction of MTT to purple formazan can be used to assess the viability of cells. PC12 cells were seeded into 96-well plates at the density of 5×10^3 cells which were enough for 16 h followed by pretreatment with different concentrations (0–2 560 µg/mL) of MEA for 72 h. A total of 90 µL of FBS-free medium and 10 µL of 5 mg/mL MTT in PBS were mixed to every well. After incubation for 4 h at the temperature of 37 °C, the formazan crystals were dissolved with 100 µL of 10% SDS (sodium dodecyl sulfate) containing 0.01 M hydrochloric acid at indoor temperature overnight. By using a microplate reader (Molecular Devices, US), the absorbance can be tested at 550 nm. Cell viability was measured to be in relative with the control.

Neurite outgrowth stimulation assay

Cells were cultivated in 24-well plates at the density of 5×10^3 cells each well and then followed by treatment with fresh MEA at various concentrations (12.5–200 µg/mL) (w/v) in the intact F-12 K medium. The cells that treated with 50 ng/mL of NGF-7S (the optimum concentration for neuritogenesis in PC12 cells) were served as a positive control, and the cells in complete F-12 K medium without treatment alone were used as a negative domination. After incubation for 72 h, the cells in randomly chosen fields were observed under examination by using an inverted light microscope (Olympus).

5 random selected areas (100–200 cells/well) were tested under an inverted microscope (Olympus) in each well and neurite length was tested in more than 50 cells in

randomly picked areas by using image processor system Image-Pro Insight (Media Cybernetics, MD). Neurite that was twice or more than the size of the cell body's diameter can be regarded as a neurite-bearing cell. Furthermore, the percentage of neurite-bearing cells (%) was evaluated and determined by scoring the proportion of neurite-bearing cells which were divided by cells that's in relative to the cells in one field.

Immunofluorescence staining of neurofilaments

The immunofluorescence assay was based on the Schimmelpfeng et al. [24] with slight modifications. Briefly, the PC12 cells were plated in a 24-well plate at the density of 5×10^3 cells each well and were treated with MEA for 72 h in a 5% of CO₂-humidified incubator. After being fixed with 4% of paraformaldehyde for half an hour, the cells were incubated with 200 anti-neurofilament antibodies (1:160; Sigma) overnight at 4 °C and then labelled with FITC-conjugated goat anti-rabbit IgG (1:80; Santa Cruz) for 1 h. The cells were mounted with ProLong® Gold antifade reagent with 4-6-Diamidino-two-phenylindole (DAPI) (Life technologies Corporation) at last. Slides can be seen with the help of Leica DM1000 microscope through fluorescence illumination with FITC and DAPI filters.

Western blotting

A total of 3×10^6 cells were seeded in a 60-mm dish for 16 h and then followed by the treatment with MEA or NGF (50 ng/mL) for 72 h. Whole cell lysates were prepared with RIPA as stated before and the protein's concentration was decided by Bradford assay. Appropriated amount protein in total cell lysates were isolated on a sodium dodecylsulfate-polyacrylamide gel and then transformed to polyvinylidene difluoride (PVDF; Bio-Rad) membranes, and they were blocked in Tween 20 buffer/Tris-buffered saline which contained 3% BSA, 2 mM EDTA and 20 mM NaF for 1 h at the indoor temperature and then cultivated with the main antibodies (TrkA, ERK, Akt and corresponding phosphorylated antibodies as well as anti-neurofilament 200) diluted in blocking buffer all over the night at 4 °C. The membrane was further cultivated with HRP-conjugated secondary antibody at 37 °C for 1 h after cleaned for 3 times with TBST buffers. With the help of an enhanced chemiluminescence detection system (Bio-Rad, CA) and equipped with densitometry analysis, the protein bands can be visualized.

Measurement of NGF levels

The concentration of extracellular NGF of MEA-treated cells were measured by using ChemiKine NGF, Sandwich

ELISA Assay Kit (Merck Millipore, MA, USA) in accordance with the manufacturer's instruction. Briefly, the PC12 cells were planted at a density of 1×10^4 cells each well in a 96-well plate and cultivated without or with NGF (50 ng/mL) and MEA (20–100 $\mu\text{g/mL}$) for 72 h. Then the supernatant was collected and the cells' cultural supernatant centrifuged at $1500 \times g$ for 15 min and supernatant maintained at 0–4 °C which was prior to assay. The samples were diluted with sample diluent at a ratio of 1:2 (v/v). By using a Microplate Reader (Molecular Devices, US), the absorbance can be tested at 450 nm.

Treatment with specific inhibitors of signaling pathways

The special inhibitors, including the PI3K inhibitors (LY294002), the MEK inhibitors (U0126, PD98059) and the TrkA inhibitors (GNF5837), were used to block the signal pathways involved in neuritogenesis. All the inhibitors were prepared in DMSO and stored at the temperature of -20 °C in the dark. The cells were incubated without or with a special inhibitor for 1 h, and then followed by the optimum concentration of MEA for 72 h or the treatment with 50 ng/mL (w/v) of NGF to scoring the neurite-bearing cells.

Statistical analysis

All experiments were carried out in triplicate. All the data were put as the mean $\pm$ standard deviation (SD) and all analyzes were done by using SPSS 19.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was applied to determine the great differences between the groups. The multiple range tests (DMRT) $p < 0.05$ of Duncan was regarded to be of significant difference.

Results and discuss

The cells viability and MEA's cytotoxic effects on PC12 cells

After treatment with MEA, the cell's viability decreased slowly in a concentration dependent way. The viability of cells' treatment with MEA at a low concentration (< 320 $\mu\text{g/mL}$) did not have significant change. Moreover, MEA could promote the cell's proliferation at the concentration of 5 $\mu\text{g/mL}$. However, MEA showed cytotoxic effect on PC12 cells when the concentration was more than 320 $\mu\text{g/mL}$ [Fig. 2(A)]. The IC_{50} of MEA was about 300 $\mu\text{g/mL}$ [Fig. 2(B)].

Mushrooms showed great potential to serve as a poly pharmaceutical drug as they could provide different kinds of

bioactivities and chemical contents [25]. Water extraction, the most effective method to obtain natural products, was usually applied in traditional Chinese medicine preparation, which was one of the most effective methods to get active agents from natural products in traditional Chinese medicine (TCM). The extracts' components were not the same when it was extracted by the solvents of different polarity. According to Wong et al., the extracts of *Hericium erinaceus* (Bull. Fr.) Pers. mycelium and fruiting body induced by neurite outgrowth of neuronal cells NG108-15 in test tube [26]. However, the neurite outgrowth of PC12 cells can be promoted by the extractions of ethanol of *H. erinaceus* and can also enhance NGF's secretion from 1321N1 human astrocytoma cells and NGF mRNA expression [27]. In addition, *Lignosus rhinocerotis* (Cooke) sclerotium's extractions and crude polysaccharides stimulated neuritogenic activity without stimulating the production of NGF in PC12 cells [28].

Water extraction contained polysaccharide and bioactive secondary metabolites such as sterols and triterpenes, which could enhance nerve growth factors (NGF)-induced neuritogenesis [29]. Moreover, there was evidence that bioactive compounds (alkaloids, flavanoids and phenolic compounds) which were from herbs and extracted with ethanol had been proved to be better activities than aqueous extractions. However, these bioactive components had better antioxidant effects than neuritogenic effects [30]. Furthermore, aqueous extraction was considered to have lower cytotoxicity effects than ethanol extraction [31]. Based on the above analysis, water extraction was selected to get MEA in this study. The fruiting bodies of *M. importuna* were soaked with 95% EtOH for 24 h before water extraction in order to remove alcohol soluble substances.

Neurite stimulation activity assay of MEA

As can be seen in Fig. 3(A), after 72 h of incubation, a eye-seeing neurite outgrowth appeared in PC12 cells, and the percentage of neurite-bearing cells that treated with MEA at the concentration range 20–160 $\mu\text{g/mL}$ was increased greatly ($p < 0.05$) compared with the negative effect (cells in intact F-12 K medium alone). The optimum concentration of MEA that stimulated neuritogenesis with $12.4 \pm 1.4\%$ of neurite-bearing cells was 80 $\mu\text{g/mL}$, which was far away below the IC_{50} value of the cytotoxic activity of MEA, and $15.1 \pm 2.1\%$ of neurite-bearing cells induced by NGF were at the concentration of 50 ng/mL, which had been confirmed as the optimum concentration of NGF for neuritogenesis in PC12 cells [Fig. 3(B)]. Moreover, the percentage of PC12 cells that treated with different concentrations of NGF and optimum concentration of MEA [80 $\mu\text{g/mL}$ (w/v)] appeared a rising in the number of

Fig. 2 Results of MEA on the cells' viability of PC12 cells. **(A)** The PC12 cells were treated with aqueous extracts of *M. importuna* (MEA) at different concentrations from 0 to 2,560 μg/mL for 72 h. Compared with the respective control 100%, findings were shown as

mean $\pm$ standard deviation ($n = 3$). $*p < 0.05$. **(B)** The IC_{50} value for MEA in PC12 cells was determined with MTT method. The linear part of the percentage inhibition (% in h) curve was carried out to obtain the intercept on the x-axis ($y = 0$) of the regression line

Fig. 3 Neurite outgrowth stimulation activity assay. **(A)** The percentage of neurite-bearing cells of PC12 cells were incubated with different concentrations of MEA from 5 to 160 μg/mL. PC12 cells in medium were in negative control while the cells treated with 50 ng/mL (w/v) NGF were in positive control. **(B)** In the cell line PC12, the

percentage of neurite-bearing cells treated with combination of various concentrations NGF (ng/mL) and 20 μg/mL (w/v) of MEA. **(C)** Expression of NF-200 protein detected by Western blotting. Data were expressed as mean $\pm$ standard deviation ($n = 3$). Means with different alphabets showed significant difference ($p < 0.05$)

neurite-bearing cells in comparison with the cells that treated with optimum concentration of MEA only and also the same concentration of NGF alone. The percentage of neurite-bearing cells that treated with optimum concentration of MEA was up to $19.2 \pm 1.3\%$ (best neurite outgrowth activity) after addition of 30 ng/mL of NGF. In addition, there were significant differences between binary systems of NGF + MEA and the optimum concentration of NGF. Furthermore, NGF and MEA could increase the expression of NF-200 [Fig. 3(C)].

As we can see in Fig. 4, the neuritogenesis was stimulated by NGF and MEA and the expression of NF-200 increased significantly. Immunostaining of neurofilaments confirmed that the neuritogenesis was stimulated by NGF and MEA. Neurofilaments were changed to green by anti-NF-200 antibody labelled with FITC while PC12 cells nuclei were stained blue with DAPI.

MEA had neuritogenic activity and increased the expression of NF-200 protein. Our data proved that the combination of MEA and a lower concentration of NGF

Fig. 4 Neurofilament stains on PC12 cells. The anti-neurofilament 200 kD labeled with FITC staining for neuronal cells, green; DAPI staining for nuclei, blue. A, B, C, D represent PC12 cells treated with medium, 50 ng/mL (w/v) NGF, 80 µg/mL (w/v) MEA, and combination of 80 µg/mL (w/v) of MEA and 30 ng/mL (w/v) NGF, respectively. Scale bars represent 50 µm. Arrows indicated expression of NF-200 protein. (Color figure online)

were able to have a better stimulation of neurite outgrowth compared with the stimulation activity with a little higher concentration of NGF at 50 ng/mL (w/v) alone. As extracellular NGF concentration in supernatants of MEA-treated PC12 cells was not increased, it was confirmed that MEA contains some substances regarded as substitutes for NGF (NGF-mimicking activity), but have no effect on promoting the biosynthesis of NGF in PC12 cells.

Elucidation of the involvement of NGF-responsive pathway involved in MEA-stimulated neuritogenesis in PC12 cells

The concentration of extracellular NGF of PC12 cells without treatment (negative control) was 53.4 ± 3.8 pg/mL [Fig. 5(A)], while this index in 50 ng/mL of NGF-treated cells (positive control) was 317.4 ± 24.8 pg/mL, which was nearly sixfold increase compared to the negative control. However, no significant differences were found in the content of extracellular NGF in MEA-treated cells.

The NGF responsive pathway, TrkA, MEK/ERK and PI3K/Akt pathways were inspected. The MEK/ERK1/2 inhibitors (namely U0126 and PD98059) and the PI3K/Akt inhibitors (namely LY294002) are significantly blocked by NGF-, MEA- and NGF + MEA-stimulated neuritogenesis. Noteworthy, the TrkA inhibitors GNF5837 had been significantly blocked by the NGF- and NGF + MEA-

stimulated neuritogenesis, but had almost no effect on the percentage MEA-stimulated neurite-bearing cells [Fig. 5(B)].

The activity of key proteins of NGF-responsive pathway involved had been investigated in PC12 cells. As shown in Fig. 5(C), the activation of phosphorylation of TrkA was greatly increased following by the NGF addition and MEA had a similar weaker effect. Additionally, both MEA and NGF could increase ERK and Akt phosphorylation, an essential step in the NGF-responsive pathway. Thus, the potential neuritogenic activity of MEA was through specific modulation of phosphorylation of TrkA, ERK and Akt.

Particular attention was drawn to GNF5837, the TrkA inhibitors, which had significantly blocked the NGF- and NGF + MEA-stimulated neuritogenesis, but had almost no effect on the percentage MEA-stimulated neurite-bearing cells. TrkA-related tyrosine kinase (TrkA), a cell surface transmembrane receptor tyrosine kinase for NGF [32], which led to a uninterrupted activation of phosphoinositide-three-kinase/protein kinase B (PI3K/Akt) and extracellular signal-regulated kinases (ERKs) which were necessary for the outgrowth of neurite. Neuritogenic effects of some compounds in MEA weren't completely resisted by GNF5837 (the TrkA inhibitors), which were not only because of the classical neurotrophic factors. Conjecture can be made that MEA-induced neurite outgrowth potentiated wasn't completely Trk-dependent other than NGF was completely dependent on the phosphorylation of TrkA, and NGF-like compounds may be contained in MEA.

It has been proved that the MEK/ERK1/2 signaling pathway can cascade for NGF to stimulate neuritogenesis in PC-12 cells efficiently [33]. This signal transduction pathway played a role by activating the expression of anti apoptotic proteins and verified neuritogenesis stimulated by hot aqueous extracts of many kinds of medicine mushrooms in vitro [34]. Additionally, the MAP (mitogen-activated protein), family members of kinase, included extracellular signal-regulated kinase was involved in neural apoptosis [35]. by directly inhibiting the production of apoptotic proteins to exert effects. Furthermore, mushroom extracts may have neuroprotective effects by inhibiting the apoptosis of nerve cells. In summary, mechanisms of MEA in promoting neuritogenesis in PC12 cells can be summarized into one overall argument that some bioactive components in MEA can exhibit NGF-mimicking activity and other agents may enhance the activity of NGF. The extracts components were not exactly the same with the solvents of different polarity.

Our findings suggested that hot water extracts from the fruiting bodies of *M. importuna* (MEA) possessed neuritogenic activity without cytotoxic effect at a low concentration (< 320 µg/mL). The bioactive component(s) in

Fig. 5 Elucidation of the involvement of NGF-responsive pathway involved in MEA-stimulated neuritogenesis in PC12 cells. **(A)** Extracellular NGF concentration in supernatants of NGF- or Mea-treated PC12 cells. Cells were cultivated without or with MEA (10–160 μg/mL), NGF (50 ng/mL) for 72 h. Cells in intact F-12 K medium played a negative effect while cells treated with 50 ng/mL of NGF served as a positive control. Data were output in the method of mean $\pm$ standard deviation ($n = 3$). Various alphabets' ways indicated significant differences ($p < 0.05$). **(B)** The special inhibitors' effects of TrkA, MEK/ERK1/2 and PI3K/Akt on NGF- or Mea-

stimulated neuritogenesis. Cells were exposed with GNF5837, U0126, PD98059 and LY294002 for 1 h before the treatment with NGF (50 ng/mL) or MEA (80 μg/mL). The treatment of medium can serve as a negative control while the cells treated with 50 ng/mL of NGF worked as a positive control. In each treatment group, a control without inhibitors was used. Compared with the respective controls, the data were put as mean $\pm$ standard deviation ($n = 3$). * $p < 0.05$. **(C)** The levels of phosphor- or total TrkA, ERK and Akt protein detected by Western blotting

MEA may mimic, enhance the NGF's neuritogenic activity and induce neuritogenesis in PC12 via NGF responsive pathway, PI3K/Akt and MEK/ERK1/2 signal pathway. The potential neuritogenic activity of *M. importuna* provides us the evidence for the use of morels as a traditional medicine rather than being already eaten as food. Delightfully, morels had neuritogenesis effects might be considered as a dietary supplement that could prevent or treat conditions occurred due to senescence of neurons.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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