

# Effect of Honokiol on culture time and survival of Alzheimer's disease iPSC-derived neurons

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## Article Info



### Article Type:

Original Article

### Article History:

Received: 7 Nov. 2022

Revised: 19 May 2023

Accepted: 22 Jul. 2023

ePublished: 5 Aug. 2023

### Keywords:

Episomal plasmid, Induced pluripotent stem cells, PBMCs, Neuronal differentiation, Neurotrophic agent, Honokiol

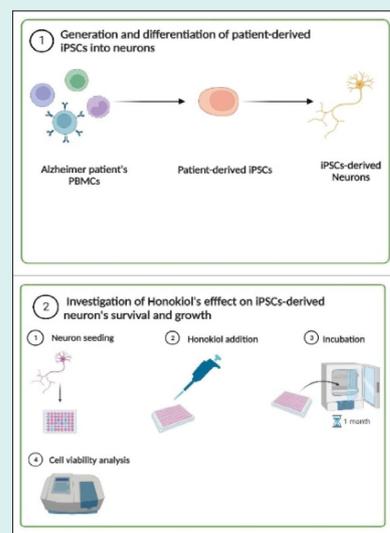
## Abstract

**Introduction:** Patient-derived induced pluripotent stem cells (iPSCs) have been widely used as disease models to test new therapeutic strategies. Moreover, the regenerative potential of stem cells can be improved with the use of biologically active compounds. Our study was designed to explore the effect of honokiol, a small polyphenol molecule extracted from *Magnolia officinalis*, on the survival and culture time of iPSC-derived neurons from a sporadic Alzheimer's disease (AD) patient. This study aimed to generate iPSCs from peripheral blood mononuclear cells (PBMCs) of an AD patient using episomal plasmids with a nucleofactor system and differentiate them into neurons. These iPSC-derived neurons were used to investigate the effect of honokiol extracted from *M. officinalis* on their survival and long-term cultures.

**Methods:** iPSCs were generated from PBMCs of an AD patient by introducing Oct-3/4, Sox2, Klf4, L-Myc, and Lin28 using Nucleofector™ Technology. Differentiation of neurons derived from iPSCs was carried out using inducers and recognized by biomarkers. The viability of iPSC-derived neurons with the addition of honokiol extracted from the bark of *M. officinalis* was determined by the MTT analytical kit.

**Results:** iPSCs were generated by reprogramming AD patient-derived PBMCs and subsequently converted into neurons. The survival and growth of iPSC-derived neurons were significantly enhanced by adding honokiol in the experiment conditions.

**Conclusion:** AD iPSC-derived neurons had a high viability rate when cultured in the presence of honokiol. These results have shown that AD iPSC-derived neurons can be an excellent model for screening neurotrophic agents and improving the conditions for long-term cultures of human iPSC-derived neurons. Honokiol proves to be a potential candidate for cellular therapeutics against neurodegenerative disorders.



## Introduction

Their abilities to proliferate and differentiate make stem cells powerful medicinal products. Recent researches have focused on stem cell therapy for the treatment of various diseases such as neurological conditions, diabetes, myocardial infarction and leukemia. Induced pluripotent stem cells (iPSCs) resemble embryonic stem cells (ESCs) but have no effects on the host immune system and avoid

ethical issues.<sup>1</sup> Somatic cells are harvested from the patients and treated with factors responsible for reprogramming to obtain iPSCs,<sup>2-4</sup> which can be further differentiated into various cell types in the body. In addition to transplantation therapy, iPSCs can be utilized for disease modeling and drug screening. Common sources of patient-derived cells for reprogramming include fibroblasts and peripheral blood mononuclear cells (PBMCs). iPSCs were first



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generated from skin fibroblasts; however, skin biopsy is invasive and might cause visible scars.<sup>5</sup> As an alternative, mononuclear cells can be harvested from peripheral blood of the patient. Blood draw is minimally invasive and routinely performed.<sup>6</sup> Numerous methods to reprogram somatic cells are available with specific advantages and disadvantages. The initial approach for generating iPSCs was integration of exogenous DNA into the host genome using retrovirus<sup>7</sup> or lentivirus<sup>8</sup> with high reprogramming efficiency. However, these methods might increase the risk of tumor formation and insertional mutagenesis, resulting in safety concerns in their application in a clinical setting.<sup>9,10</sup> Safer techniques have been investigated such as episomal plasmids, Sendai virus vectors, electrical impulses (Nucleofector and Neon transfection system), chemical-based methods (Lipofectamine 3000) or elastin-like polypeptide (ELP)-based transfection and synthetic messenger RNA.<sup>10-12</sup> Among these techniques, nucleofection has been demonstrated as a highly efficient gene transfection approach.<sup>13</sup>

The advent of iPSCs has enabled the generation of patient-specific Alzheimer's disease (AD) models for pathogenesis studies and drug screening. AD is a form of dementia with cognitive and functional decline that can negatively affect the daily life of patients and eventually result in death. Biological hallmarks of AD were identified in mid-1980s as extracellular amyloid- $\beta$  (A $\beta$ ) plaques and intracellular neurofibrillary tangles comprised of hyperphosphorylated and aggregated tau proteins.<sup>14</sup> Patients can be categorized into early-onset AD, which is linked to mutations in PSEN1, PSEN2 and A $\beta$ PP genes, and late-onset AD, which is less specific with no gene mutations.<sup>15</sup> The neuroprotective effects of many antioxidants have been explored in iPSC-derived models of several neurodegenerative disorders, including AD. According to the oxidative stress theory of neuronal degeneration, the excess of free radicals results in neuronal death by means of increased mitochondrial stress.<sup>16</sup> Induced by oxidative damage, mitochondrial defects associated with energy metabolism can lead to the disruption of calcium homeostasis, which has been observed in neurodegenerative diseases such as AD and Parkinson's disease.<sup>16</sup> Persistent elevation of intracellular calcium could cause severe phenotypes in neurons, eventually leading to neuronal death and degeneration.<sup>17</sup> Targeting oxidative stress, therefore, provides an important approach to the prevention and treatment of neurodegenerative disorders. The AD brain is known to have substantially low antioxidant enzymatic activities, which makes it more prone to toxicity caused by A $\beta$ .<sup>18</sup> Therefore, antioxidant agents have gained interest in the search of novel drugs for AD treatment. Natural antioxidants exhibit neuroprotective effects through a range of mechanisms such as scavenging reactive oxygen species (ROS), interacting with transition metal ions, altering enzymatic activities, modulating intracellular signaling

and gene expression.<sup>16</sup> Several groups have used AD iPSC-derived neurons to discover neuroprotective effects of natural antioxidants extracted from plants such as N-butyridenephthalide,<sup>19</sup> apigenin,<sup>20</sup> Thymoquinone.<sup>21</sup> Balez et al found that apigenin, an antioxidant polyphenol found in parsley, artichoke and celery, could increase viability and ameliorate disease phenotypes such as neurite shortening and hyperexcitability in AD neurons.<sup>20</sup>

Honokiol, which is an abundant polyphenol in the bark of *M. officinalis*, exhibited neuroprotective capacity as an antioxidative, anti-excitotoxic, anti-neuroinflammatory and anti-A $\beta$  agent.<sup>22</sup> Intravenous administration of honokiol reduced lipid peroxidation and ROS production in the rat brain with focal cerebral ischemia-reperfusion injury (CIRI),<sup>23</sup> and oral administration prevented oxidative stress in N-methyl-D-aspartic acid-treated mice.<sup>24</sup> In CIRI mice's microglia treated with honokiol, nuclear factor- $\kappa$ B activation, which plays a crucial role in inflammation, was suppressed through the reduction of p65 subunit level.<sup>25</sup> In the same study, culture medium of glial cells showed lower levels of inflammatory factors such as nitric oxide, tumor necrosis factor- $\alpha$  and RANTES protein.<sup>25</sup> Honokiol reduced cell death prompted by A $\beta$  toxicity through suppressing ROS production, Ca<sup>2+</sup> elevation and caspase-3 activation.<sup>22</sup> In addition, honokiol has been demonstrated to traverse the blood-brain barrier as well as the blood-cerebrospinal fluid barrier.<sup>26,27</sup> However, there have been no reports of neuronal survival promotion by honokiol in AD iPSC-derived neurons.

## Materials and Methods

### Materials

Four plasmids: pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, pCXLE-hUL and pCXWB-EBNA1 carrying reprogramming factors: Oct-3/4, Sox2, Klf4, L-Myc and Lin28 were supplied by Addgene (Waterdown, MA, USA). P3 Primary Cell 4D-Nucleofector™ X Kit and 4D-Nucleofector™ System were bought from Lonza (Cologne, Germany). The medium components including DMEM/F12, DMEM, Panexin NTS, 2-mercaptoethanol, fetal bovine serum (FBS), Penicillin – Streptomycin (PS) were purchased from PAN Biotech (Aidenbach, Germany). TRIzol reagent, MitoTracker® solution, phosphate-buffered saline (PBS), FGF-2 and Paraformaldehyde (PFA) were obtained from Invitrogen (Waltham, MA, USA). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA).<sup>11</sup> RevertAid First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and PCR Master Mix kit was purchased from Promega (Madison, WI, USA). Honokiol was isolated and purified from *M. officinalis* with a purity > 97%. Honokiol's purity was identified by comparison with standard purchased from Shanghai Aladdin Bio-Chem Technology (Shanghai, China). Honokiol's structure was determined based on Fourier transformed infrared (FT-IR) (Nexus 670

Thermo Nicolet Fourier Transform Infrared Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA), liquid chromatography-mass spectroscopy (LC-MS) (the Agilent 1100 Series LC/MSD Trap SL, Agilent Technologies, Santa Clara, CA, USA), and nuclear magnetic resonance (NMR) spectral analyses (Bruker Corporation, Billerica, MA, USA).

#### **Reprogramming of PBMCs to iPSCs by nucleofection**

Peripheral blood sample was donated by a 69-year old male patient with clinically and genetically characterised AD by the National Geriatric Hospital (Vietnam) after obtaining informed consent. All procedures performed in study involving participants were in accordance with the ethical standards of the institutional and/or national research committee with number 2151/QĐ-VHL.

Then the PBMCs were isolated by density gradient centrifugation using Ficoll-Paque<sup>®</sup>-1077 (Sigma Aldrich, St. Louis, MO, USA) at  $1000 \times g$  for 30 minutes and 20°C. After the isolation procedure, PBMCs were seeded in DMEM supplemented with 10% FBS, 1% PS. To generate iPSCs, PBMCs were transduced with five episomal vectors pCXLE-hOCT3/4, pCXLE-hSK, pCXLE-hUL, pCXWB-EBNA1. Briefly,  $1 \times 10^6$  cells were washed by DMEM/F12 medium and then electroporated using nucleofection kit P3 solution and Nucleofector 4D, program EO-100 (Lonza, Cologne, Germany). Transfected cells were plated in DMEM high-glucose medium. Cells transfected with GFP were used as controls. On day 7, the medium was replaced by the iPSC-specific medium (DMEM/F12, 20% pannexin, 4 ng/mL FGF-2, 100 nM  $\beta$ -mercaptoethanol, 1% PS). After 15-20 days, colonies which have ES-like appearance were observed under a microscope. Between day 20-30 post-transduction, cells were manually picked based on their morphology. iPSCs were cultured on 6-well plates coated with Matrigel and in mTESR1 medium. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>, fed daily with medium until confluence reached 80-90%.

#### **Immunocytochemistry analysis**

Cells were first fixed with 4% paraformaldehyde for 5 minutes at room temperature. After washing with PBS (Thermo Fisher Scientific, Waltham, MA, USA), cells were incubated with 0.2% Triton X-100 in PBS for 30 min at room temperature. Subsequently, the iPSCs were incubated with antibodies for the pluripotency markers Oct-3/4 (Oct-3/4 is conjugated to Alexa Fluor<sup>®</sup> 594 (sc-5279, AF594)); Nanog (1E6C4) is conjugated to Alexa Fluor<sup>®</sup> 488 (sc-293121 AF488)); and nerve cells were incubated with antibodies MAP-2 (MAP-2 antibody is conjugated to Alexa Fluor<sup>®</sup> 594 (sc-32791, AF 594)) overnight at 4 °C. After the cells were rinsed with PBS for three times, nuclei were counterstained with DAPI. Cells were observed using a Nikon Ti E Eclipse microscope equipped with a Ti-TIRF illumination unit and confocal system.

#### **Karyotype analysis**

iPSCs were treated with KaryoMAX<sup>®</sup> Colcemid<sup>™</sup> Solution (Thermo Fisher Scientific, Waltham, MA, USA) and using standard cytogenetic procedures. Chromosomes were analyzed by G-banding and were carried out by Hanoi Obstetrics & Gynecology hospital.

#### **Neural induction**

Neural progenitor cells (NPCs) were generated from the patient's iPSCs using PSC Neural Induction Medium (NM) (Thermo Fisher Scientific, Waltham, MA, USA). Initially, iPSCs were seeded in 6-well plates and when the confluency of iPSCs reached to 20%, the cells had its medium replaced with neural induction medium (NIM) (1:1 vol/vol mixture of DMEM/F12 and neurobasal medium, 1× N-2 supplement, 1× B-27 supplement, 1× nonessential amino acids [NEAA], 2 mM L-glutamine, 50 U/mL penicillin/streptomycin, 100  $\mu$ M  $\beta$ -mercaptoethanol, retinoic acid 5  $\mu$ g/mL insulin), which was supplemented with 5 ng/mL basic fibroblast growth factor (bFGF). NIM was changed every 2 days. Neural cells were cultured in neural maintenance medium (1:1 vol/vol mixture of DMEM/F12 and neurobasal medium, 2 mM L-glutamine, 50 U/mL penicillin/ streptomycin, 1× N-2 supplement, 1× B-27 supplement, 1× NEAA) and supplemented with 10 ng/mL epidermal growth factor and 10 ng/mL bFGF.

#### **DPPH radical scavenging assay**

The antioxidant property of honokiol was investigated using the DPPH scavenging assay adapted from a method previously described.<sup>28</sup> Honokiol was diluted in DMSO at various concentrations to prepare a sample solution. Ascorbic acid was used as a reference standard. The sample solution and ascorbic acid (9  $\mu$ L) were added to 171  $\mu$ L of DPPH solution (0.24 mg/mL). After a 20-minute incubation, the absorbance of the reaction mixture was measured at 517 nm by a UV-Vis spectrometer. The percentage of inhibition of DPPH radicals was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_0 - A_1) / A_0 \times 100$$

where A<sub>0</sub> is the absorbance of control, and A<sub>1</sub> is the absorbance of the test or standard sample.

#### **Comet assay**

The in vitro genotoxicity of honokiol was evaluated using the alkaline comet assay based on a protocol previously described.<sup>29</sup> After respective treatments with honokiol (10  $\mu$ g/mL) or H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M), the neurons were collected and washed with PBS before being resuspended in low melting agarose 1%. The suspension was spread on microscope slides which had been coated with a thin layer of normal melting agarose. The slides were refrigerated for 10 minutes to allow the low melting agarose to solidify,

followed by overnight immersion in the lysis buffer at 4 °C. The slides were then immersed in the alkaline buffer for 1h, and electrophoresed for 30 minutes at 4 °C. After that, the slides were stained with DAPI and observed using a Nikon Ti E Eclipse microscope equipped with a Ti-TIRF illumination unit and confocal system.

### Cell viability assay

AD iPSC-derived neurons were grown in 48-well plates at 24 hours. The medium was replaced by 200 µL of the formulation at pure honokiol concentrations of 0.1-10 µg/mL followed by incubation for 1 month at 37 °C and culture medium was changed every 3 days. Cell viability was assessed by MTT assay (Promega, Madison, WI, USA) according to manufacturer's instructions. Cell viability was determined using the following equation:

$$\% \text{ Viability} = 100\% - \frac{OD_{\text{reagent}} - OD_{\text{day0}}}{OD_{\text{negative control}} - OD_{\text{day0}}}$$

### Statistical analysis

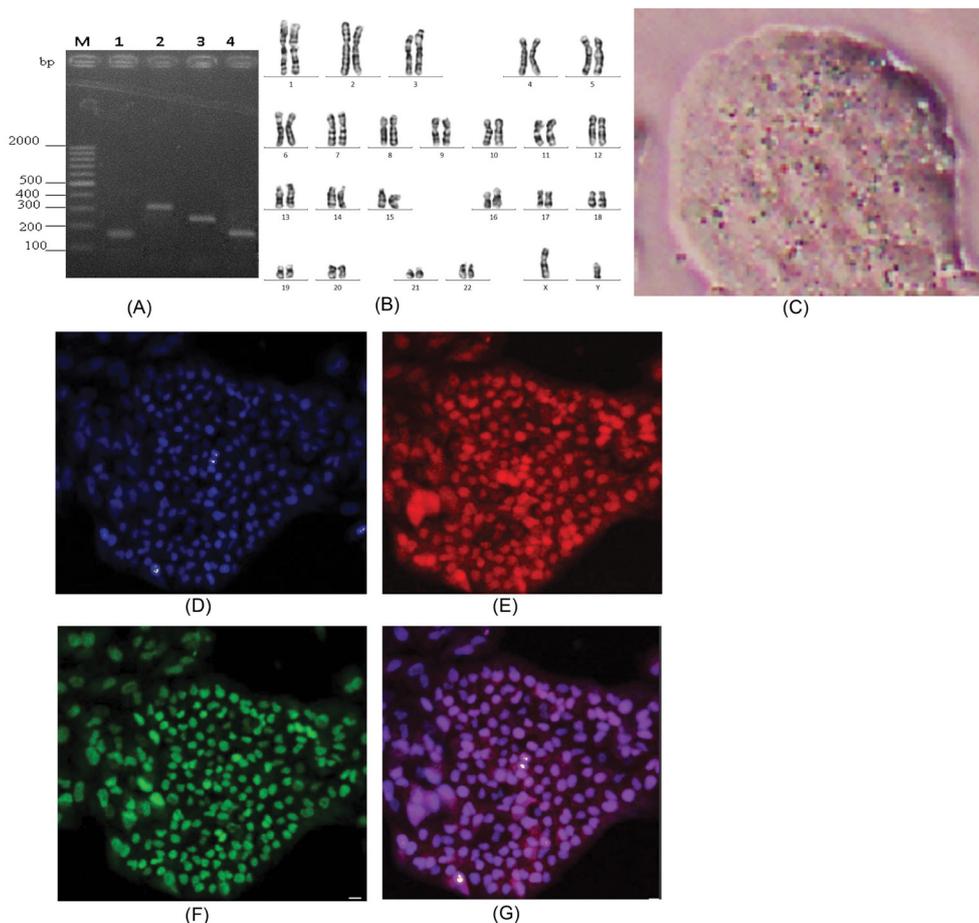
Data were expressed as the mean ± standard deviation (SD). The data were statistically analyzed using Student's t test. The differences between the samples were considered to be significant when *P* value is < 0.05.

## Results

### Generation of iPSCs from PBMCs

In our research, PBMCs were reprogrammed into iPSCs by using a non-integrating method. Nucleofector with the plasmid DNA cocktail which contained the four vectors in equimolar amounts. The colonies were formed after 3 weeks of transfection with ESC-like characteristics (Fig. 1C). Thus, the expression of pluripotency marker genes by electrophoresis from wells 1; 2; 3; 4 showed clear DNA bands such as Oct-3/4 (144 bp), c-MYC (328 bp), Nanog (287 bp), Sox2 (151 pb) (Fig. 1A). These results supported the roadmap to reprogram and provided reliable evidence of the successful reprogramming process.

The karyotype of the iPSC line was examined using Giemsa-banding, which demonstrated a normal diploid 46, XY karyotype, with no observable abnormalities (Fig. 1B). Immunofluorescence analysis confirmed the expression of pluripotency markers Oct-3/4 (Fig. 1E) and Nanog (Fig. 1F), demonstrating the pluripotent nature of the iPSC line. Overall, these results showed that we successfully generated iPSCs from PBMCs by transfection with Yamanaka reprogramming factors in a feeder-free medium.



**Fig. 1.** Characterization of induced pluripotent stem cells from peripheral blood mononuclear cells: (A) Expression of pluripotency marker genes: M: DNA marker, 1: Oct-3/4, 2: c-MYC, 3: Nanog, 4: Sox2. (B) Images of karyotyping of iPSC line; (C). iPSC colony; (D), (E), (F), (G) Immunofluorescence staining of pluripotency markers: (D) DAPI, (E) Oct-3/4, (F) Nanog, (G) Merged.

### Generation and characterization of neurons from iPSCs

After deriving neuronal progenitor cells from hiPSCs, we induced the NPCs to differentiate into neural cells by culturing them in the neuron differentiation media. The cells were subsequently stained for MAP-2, a marker of neuronal differentiation. We found that the induced cells were stained positive for the neuronal marker (Fig. 2), implying that NPCs had differentiated into neurons.

### Analysis of honokiol's radical scavenging activity and genotoxicity

Honokiol with molecular formula  $C_{18}H_{18}O_2$  and molecular weight of 266 g/mole was isolated from *M. officinalis* and its purity was confirmed by high-performance liquid chromatography (single peak) as well as by  $^1H$  and  $^{13}C$  NMR spectra and high resolution electron ionization mass spectrum (Supplementary file 1).

DPPH scavenging assay was conducted to investigate honokiol's ability to inhibit free radicals. As shown in Fig. 3A, honokiol and ascorbic acid (reference standard) showed an increase in the inhibition of DPPH radicals in a concentration-dependent manner, reaching 62.75 and 80.92% inhibition at the concentration of 100  $\mu g/mL$  respectively.

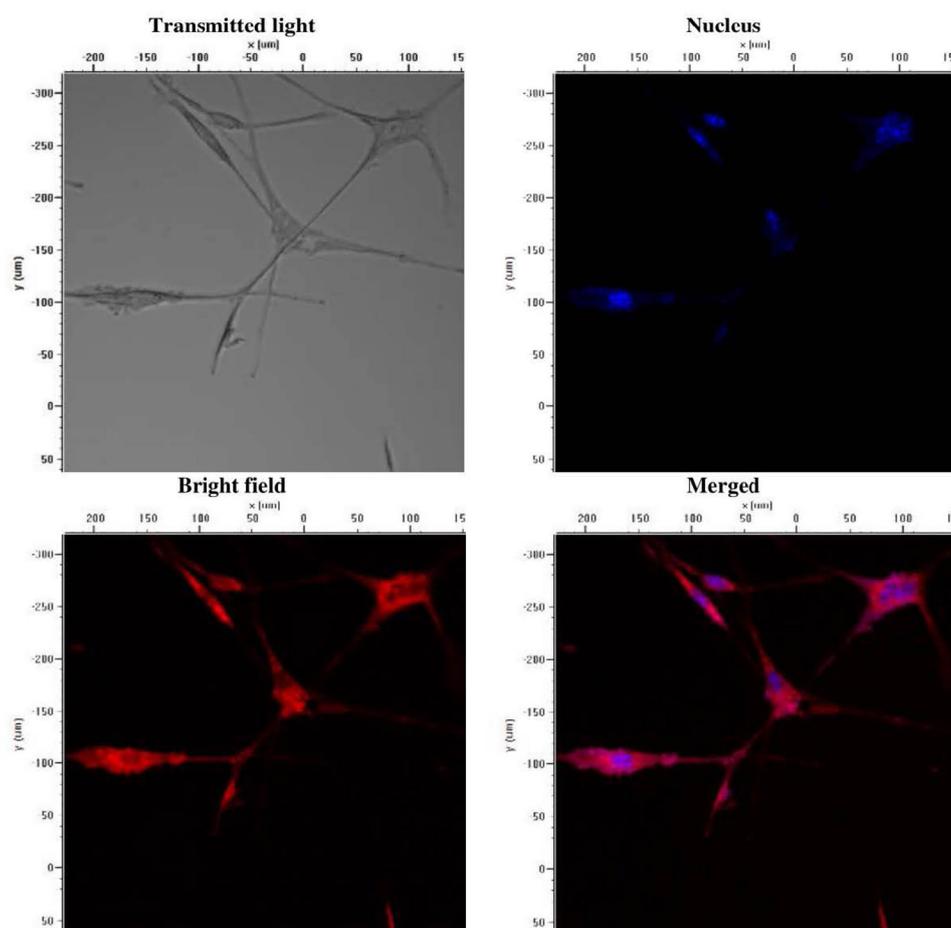
The IC50 values of honokiol and ascorbic acid were

found to be 75.66 and 53.98  $\mu g/mL$  respectively.

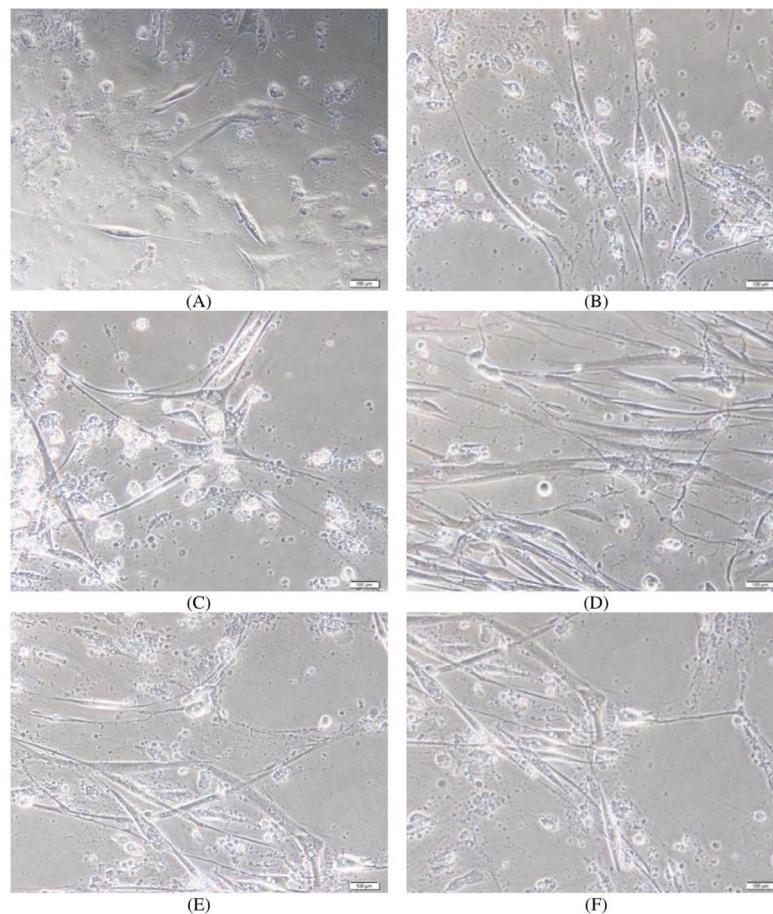
To confirm whether honokiol can induce genotoxicity in the iPSC-derived neurons, the comet assay was performed (Fig. 3B). There was no formation of tails (damaged DNA) in the honokiol-treated cells, which were similar to control cells. In contrast,  $H_2O_2$ -treated cells showed a significant increase in tail DNA. These results indicate that at the concentration of 10  $\mu g/mL$ , honokiol is not genotoxic to the iPSC-derived neurons.

### Effect of honokiol on the survival and culture time of iPSC-derived neurons

The results (Fig. 4) showed that honokiol enhanced the viability of AD iPSC-derived neurons in a dose-dependent manner (Figs. 4 and 5). After 35 days of culture, the control sample without honokiol had a high number of dead neurons. However, samples treated with different concentrations of honokiol had a lower number of dead neurons. Especially at the concentration of honokiol 5  $\mu g/mL$ , the nerve cells still developed normally compared to other concentrations. But at higher concentrations, the number of viable cells began to decrease. The above observations are shown in Figs. 4 and 5. In the honokiol-treated samples, the neurons had longer neurite outgrowth than the control samples (Fig. 4). At the concentration of



**Fig. 2.** Immunofluorescence characterization of Alzheimer's disease iPSC-derived neurons stained with DAPI (blue) and MAP-2 (Red).



**Fig. 3.** Analysis of honokiol's antioxidant property and genotoxicity. (A) Chemical structure of honokiol; (B) DPPH scavenging assay results; (C) Comet assay results. DNA damage is represented as tail moment. Data presented are means  $\pm$  SD; Student's t-test: ns  $P > 0.05$  versus the control group, \*\*\*  $P \leq 0.001$  versus the control group.

10  $\mu\text{g/mL}$ , the cell survival rate began to decrease. Thus, a reasonable concentration to increase neuronal survival in this experiment is in the range of 2-5  $\mu\text{g/mL}$ .

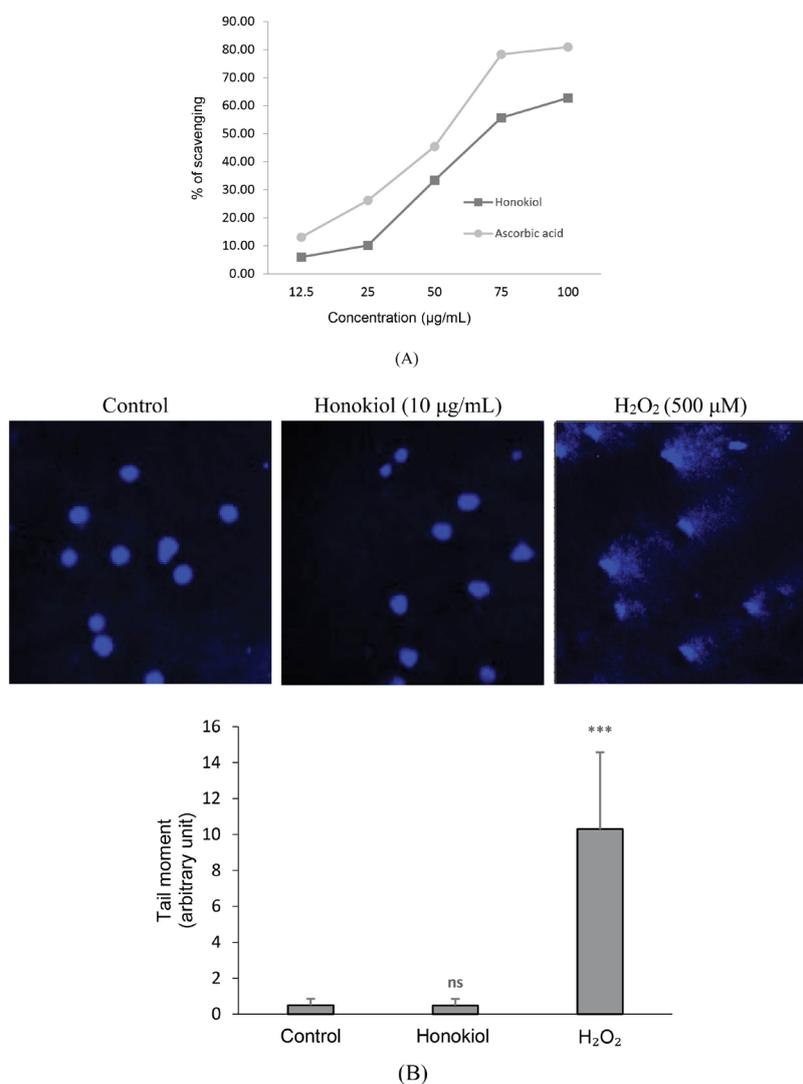
### Discussion

Thanks to their resemblance to ECSs, iPSCs have proved to be a potential tool in medical science. Since their advent, many research groups have generated iPSCs from a variety of somatic cell types, typically fibroblasts.<sup>7,30</sup> While it is clinically demanding to collect fibroblasts, peripheral blood cells are more easily obtainable as surgery is not needed to extract them.<sup>31</sup> The most fundamental distinction between the PBMCs and fibroblasts is that the former cells first have to be converted into attached form in the course of reprogramming.<sup>32</sup> In this experiment, Matrigel was utilised to support cells' attachment and facilitate adherence. Afterwards, the attached cells can proliferate into reprogrammed iPSCs. Additionally, our study adopted a transfection method using plasmids to reprogram PBMCs instead of DNA-integrative retroviral and lentiviral vectors. This approach helps us to avoid the possible risks of contaminating iPSCs with random genomic insertions of viral vectors.<sup>33</sup> Furthermore, by culturing iPSCs without feeder cells, we also ensured

that iPSCs were not adversely affected by undefined or unknown constituents present in feeder cells' medium supplements, typically fetal bovine serum or proprietary serum replacements.<sup>34,35</sup>

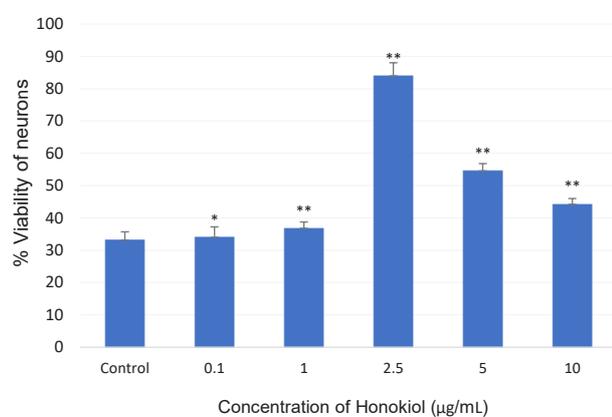
We confirmed the generation of iPSCs from PBMCs using PCR and immunohistochemistry for the expression of pluripotency marker genes, including Oct-3/4, c-MYC, Nanog, Sox2. The expression of pluripotency marker genes relates to the three sequential events of the somatic cell reprogramming process including early event (initiation phase), intermediate event (maturation phase) and final event (stabilization phase).<sup>36</sup> C-Myc is regarded as the initiation phase marker that relates to cell proliferation.<sup>36</sup> Nanog, referred as hallmark in the second transcriptional wave, is upregulated by the expressions of exogenous Oct-3/4 and Sox2. Soufi et al had also demonstrated that exogenous Oct-3/4, Sox2 and KLF4 bind to inactive DNA regions and play roles as repressor of lineage-specific genes and activator/enhancer of stemness-specific genes.<sup>37</sup> Moreover, chromosome integrity of iPS cells is a necessary condition after somatic cell reprogramming. In this study, iPSCs with a normal karyotype were competent to differentiate into different cell types.

Having established PBMC-derived iPSCs, we



**Fig. 4.** Neurite survival of Alzheimer's disease iPSC-derived neurons by honokiol after 35 days of cell culture. (A) 0.5% DMSO; (B) 0.1 µg/mL; (C) 1 µg/mL; (D) 2.5 µg/mL; (E) 5 µg/mL; (F) 10 µg/mL.

differentiated these cells into neurons and characterized them using immunofluorescence staining for MAP-2, a neuronal marker. MAP-2 is a microtubule-associated



**Fig. 5.** Effect of honokiol on cell viability of Alzheimer's disease iPSC-derived neurons after 35 days of cell culture. Data presented are means  $\pm$  SD; Student's *t* test: \*  $P \leq 0.05$  versus the control group, \*\*  $P \leq 0.01$  versus the control group.

protein that plays an essential role in the maintenance of neuroarchitecture by stabilizing microtubules, stimulating tubulin assembly and acting as substrates for protein kinases and phosphatases.<sup>38</sup> MAP-2 is predominantly found in the nervous system and is among the most plentiful proteins in brain.<sup>39</sup> MAP-2 isoforms consist of 2 groups: high-molecular weight (HMW) forms, including MAP-2A and MAP-2B, and low-molecular weight forms, including MAP-2C and MAP-2D.<sup>40</sup> The HMW forms are specifically expressed in neurons within the nervous system,<sup>38,41</sup> and therefore chosen as a specific marker for neurons. We used the MAP-2 antibody which can detect MAP-2A and MAP-2B, thus characterizing neuron formation.

In this study, we have successfully converted AD's patient-specific iPSCs into neurons.

Much of the understanding of neurological disorders has been gained from examining post-mortem neuronal tissues or animal disease models because of the limited access to patient's living neurons. The application of

iPSCs in disease modeling affords the opportunities to investigate patient-specific neuronal cells *in vitro*.

Advancement in iPSC-based neurons and differentiation protocols allow disease modeling using physiologically relevant cells. In this study, we investigated whether honokiol could improve the survival and growth of AD iPSC-derived neurons. Human iPSC-derived neurons are sensitive and require extended culturing time. Moreover, current hiPSC-derived neuron-based applications are often hindered by limitations in survival and long-term culture conditions. Therefore, modeling neuron disease using hiPSC-based approaches requires *in vitro* culture conditions to improve and to prolong the survival time. Thiry et al used a new coating substrate of cytocompatible dendritic polyglycerol amine (dPGA) for the long-term cultures of human iPSC-derived motor neurons.<sup>42</sup> The results showed an improvement in the experimental conditions of the long-term cultures of human iPSC-derived motor neurons, which could allow improved survival studies, molecular identity, electrophysiological activity and single-cell RNA sequencing.<sup>42</sup>

Neurotrophic factors have proven to be promising candidates in treating neurodegenerative disorders such as AD. However, endogenous neurotrophic factors are high-molecular mass proteins, which can hardly traverse the blood-brain barrier or can be degraded by peptidase.<sup>43</sup> Therefore, it is necessary to discover small molecules from natural products which can mimic the properties of neurotrophins. Honokiol, a natural biphenyl neolignan extracted from *M. officinalis*, has been demonstrated to traverse the blood-brain barrier as well as the blood-cerebrospinal fluid barrier.<sup>26,27</sup>

Honokiol has been shown to promote neuronal survival and outgrowth; however, studies about its neurotrophic ability have mainly focused on animal neuronal cells. These animal models may not accurately reflect electrophysiological properties of human neurons. To the best of our knowledge, our study is the first to attempt to evaluate honokiol's neurotrophic function on human iPSC-derived neurons.

Honokiol also exerted its neuroprotective effects in rats with traumatic brain injury by inhibiting the expression of APAF-1, thus preventing neurons from undergoing apoptosis.<sup>44</sup> Honokiol increased the viability of motor neurons NSC-34 cells in amyotrophic lateral sclerosis disease models both *in vitro* and *in vivo*.<sup>45</sup> In the current research, studied concentrations of honokiol resulted in a significant increase in neuron survival after 35 days, when compared to neurons without honokiol. Moreover, it was shown that honokiol exerted no genotoxicity in the iPSC-derived neurons at the concentration of 10 µg/mL. The neuroprotective effect of honokiol could be due to its antioxidant property, which was examined in this study using DPPH scavenging assay. In the present study, honokiol promoted DPPH inhibition in a concentration-dependent manner within the concentration range of

12.5-100 µg/mL.

Despite its benefits, honokiol may display neurotoxicity at high doses. Although honokiol has been shown to promote neuronal survival and outgrowth at low doses, it has also been discovered to increase neuronal death at higher doses.<sup>46</sup> This is in line with our findings about honokiol's effects at high doses.

## Conclusion

The models for researching diseases and discovering new therapies have shown to be more accurate than traditional disease models. The results showed that neurons derived from sporadic AD iPSCs were successfully generated. Pure honokiol isolated and purified from the bark of *M. officinalis* significantly affected neuronal survival and neurite outgrowth of AD iPSC-derived neurons. Thus, honokiol appears to exert neurotrophic effects on neuronal cells and to affect neurite outgrowth. These results have shown that AD iPSC-derived neurons can be an excellent model for screening neurotrophic agents and honokiol may be a potential therapeutic agent for neurodegenerative diseases.

## Acknowledgments

The authors thank Dr. Le Thi Thuy Hien, the National Geriatric Hospital (Vietnam) for her help in collection of the blood sample.

## Authors' contribution

**Conceptualization:** Duong Thi Thuy Le.

**Data curation:** Duong Thi Thuy Le, Cuong Manh Vu.

**Formal analysis:** Duong Thi Thuy Le, Cuong Manh Vu, Phuong Thi Mai Nguyen.

**Funding acquisition:** Duong Thi Thuy Le.

**Investigation:** Duong Thi Thuy Le, Cuong Manh Vu.

**Methodology:** Duong Thi Thuy Le.

**Project administration:** Duong Thi Thuy Le.

**Resources:** Duong Thi Thuy Le.

**Supervision:** Ha Hoang Chu.

**Validation:** Duong Thi Thuy Le, Cuong Manh Vu.

**Visualization:** Duong Thi Thuy Le, Cuong Manh Vu.

**Writing—original draft:** Duong Thi Thuy Le, Cuong Manh Vu, Thuy Thi Bich Ly, Nam Trung Nguyen.

**Writing—review editing:** Duong Thi Thuy Le, Cuong Manh Vu, Phuong Thi Mai Nguyen.

## Competing Interests

The authors declare they have no conflict of interest.

## Ethical Statement

Peripheral blood sample in this study was donated by a 69-year old male patient with clinically and genetically characterised AD by the National Geriatric Hospital (Vietnam) after obtaining informed consent. All procedures performed in study involving participants were in accordance with the ethical standards of the institutional and/or national research committee with number 2151/QĐ-VHL.

## Funding

This work was supported by grants from the Vietnam Academy of Science and Technology (VAST) with project number VAST02.02/20-21.

## Supplementary Materials

Supplementary file 1 contains HPLC, <sup>1</sup>H NMR, <sup>13</sup>C NMR, MS and FTIR results of honokiol as well as Table S1.

## Research Highlights

### What is the current knowledge?

✓ Alzheimer's disease iPSC-derived neurons can be an excellent model for screening neurotrophic agents and personalized medicine.

✓ Neurons derived from iPSCs often require culture conditions to improve and prolong survival *in vitro*. Therefore, substances capable of increasing cell survival need to be discovered or investigated.

### What is new here?

✓ Honokiol isolated and purified from the bark of *M. officinalis* in Vietnam significantly affected neuronal survival and neurite outgrowth of AD iPSC-derived neurons

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