The effects of the *Panax Vietnensis* ethanol fraction on proliferation and differentiation of mouse neural stem cells

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**ABSTRACT**

**Introduction.** *Panax vietnamensis* Ha et Grushv. (Ngoc Linh ginseng) – a new species recently discovered in Vietnam – has received much interest due to its rich content of saponins, including those unknown. This study assessed the effects of the Ngoc Linh ginseng extract fractions on proliferation and differentiation of cultured mouse neural stem cells. **Methods.** Whole brains were harvested from E13.5-14 Swiss mouse fetuses. Isolated cells were floating seeded to form spheroid bodies. Neurospheres were treated with one in fractions of ethanol 200-500 µg/mL, or n-butanol 200 µg/mL, or aqueous 200-500 µg/mL for 5 days. Neural stem cells could persistently generate secondary spheres. Neurospheres strongly expressed nestin, CD24 and deriving cells could differentiate into the GFAP-positive astrocyte-like cells. **Results.** Ginseng fractions significantly promoted neurosphere growth rate. Particularly, 200 µg/mL ginseng ethanol fraction significantly increased the neurosphere size (28.00±3.00%, p<0.0001) not showing degeneration to the 5th day. However, n-butanol and aqueous fraction could not sustain the sphere structure. Ginseng ethanol fraction also elevated in the G2/M proportion (28.73±0.45%, p<0.0001), up-regulated proliferation mRNA ki67 (4.605±6.48 fold-change, p<0.05), cyclA1 (12.61±4.65 fold-change, p<0.0001), cyclD1 (22.47±8.18 fold-change, p<0.0001), cycC (9.53±2.63 fold-change, p<0.0001) compared with those of the n-butanol or aqueous fraction-treated neurospheres. Shorten G0/G1 phase (47.08±0.16, p<0.0001), up-regulation of sox2 (71.25±27.24 fold-change, p<0.0001) mRNA levels indicated self-renewal effect of the ginseng ethanol fraction; however, those of n-butanol and aqueous fraction-treated neurospheres suggested an inhibiting effect on the cell proliferation. **Conclusion.** *Panax vietnamensis* extract fractions had a positive effect on the proliferation of cultured neural stem cells. The ethanol fraction at 200 µg/mL could significantly promote the growth rate while still sustained the integrity of treated spheres. **Key words:** Ethanol fraction, Mouse neural stem cells, NSCs, Panax vietnamensis, Stem cell proliferation

**INTRODUCTION**

In the Northeast and East Asian countries like Vietnam, Korea, and China, ginseng has been used thousands of years to enhance human health. *Panax* ginseng saponins were indicated improve Parkinsonian progress on animal models, cognitive performance of Alzheimer’s patients and traumatic brain injuries due to regulating the neurotrophic factor-associated pathways. Ginsenosides could promote the differentiation of neural stem cells, enhancing the neuronal fate in cultured adipose-derived stem cells. A significant source of ginseng saponins comes from popular species like *P. ginseng* C. A. Meyer, *P. notoginseng*, and *P. quinquefolium*. Recently, a new ginseng species – *Panax vietnamensis* – was found in Vietnam. New ginsenosides in *P. vietnamensis* were shown to ameliorate depression, neuronal oxidative stress and improve the cognitive performance in the mouse model. However, these studies have poorly showed the effect of ginseng extracts on the *in vitro* neuronal stem cells. Proliferating cells were discovered first in the rat brain by Altman, J. and G.D. Das. Subsequently, neural stem cells (NSCs) from both animals and humans have been extensively studied and characterized both *in vivo* and *in vitro*. In mammals, NSCs exist in both adult and embryonic brains at different developmental stages. NSCs could differentiate into three functional cell types of the nervous system. Over the past decade, there has been a rising interest in the 3D culturing method for drug screening due to its mimicking the stem cell niche in the body. Originally introduced by Reynolds and Weiss, the neurosphere culturing method has become a convenient model for screening pharmaceutical properties of substances on neural stem cells because it reduces the differentiation
possibility compared to adherent NSCs. In this study, we investigate the potential effects of *P. vietnamensis* extracts on cultured neurospheres. The proliferation and differentiation of neural stem cells were access to show the effects of *P. vietnamensis* extracts.

**MATERIALS-METHODS**

**Animal and experimental design**

This study was approved by our institutional ethical committee (Laboratory of Stem cell Research and Application, University of Science, VNU-HCM). Healthy, E13.5-15.5 pregnant Swiss mice were kept in a stable environment of 12 hours light-dark cycle in the Microventilation cage system (THREE-SHINE Inc., Korea) with ad libitum access to food and water and acclimated for 1 week before the operation.

**Plant material and preparation**

Five years of age *Panax vietnamensis* was provided by the center of Ginseng and Medicinal materials, National Institute of Medicinal Materials (NIMM), Ho Chi Minh City, Vietnam. Crude extract of *Panax vietnamensis* was prepared following the same method previously published. In brief, whole root and rhizome of the plant was air-dried and powdered. Firstly, ginseng powder was percolatively extracted using 96%, 48%, 24%, and 0% ethanol (Merck, USA), respectively. Next, the extract solutions would be evaporated at low-pressure and lyophilized to yield the crude ethanol extract (shortly regarded as “the ethanol fraction”). Lipid in the extract was eliminated by ethyl ether. Next, ethyl ether was discarded from the product and water-saturated *n*-butanol was added. The *n*-butanol was collected and lyophilized to give the *n*-butanol fraction. Deionized water was added to the remaining solution, next gathered and lyophilized to yield the aqueous fraction.

**Neural stem cells (NSCs) isolation and culture**

The NSCs isolation and culture methods in this study were repeated those in our previous study with reference to the method described by Reynolds et al. and Zheng, X.-S., et al. E13.5-15 pregnant mice were deep anesthetized by 100 mg/kg of ketamine, and 16mg/kg of xylazine and cervical dislocated. Fetal brains were isolated and homogenized into sterile PSBA solution. Brain pieces were digested with 0.025% trypsin 0.02% EDTA solution for 10 minutes at 37°C. Trypsin inhibitor (Sigma- Aldrich, St Louis, MO) was used to stop the digestion. Single cells were collected through a 70 μm Falcon® cell strainer. About 2.10⁶ cells was suspended in 5 mL of basal NSC medium (serum-free DMEM/F12 high glucose, containing 30 μg/mL EGF, 30 μg/mL bFGF, 500 IU/mL heparin, 5 mg/mL insulin, 1 mg/mL transferrin, 0.01mg/mL gentamicin) (all purchased from Sigma Aldrich, St Louis, MO), supplemented with 1X N-2 and 1X B-27 (Gibco®, ThermoFisher Scientific, USA). Cells were seeded upon the agarose-covered 25cm² culture flask (Corning, USA) to prevent adhesion and cultured at 37°C, 5% of CO₂. Medium was changed every 3 days.

**Sub-culture and sphere formation assay**

At confluence, all neurospheres or cell clumps were digested by 0.025% trypsin 0.02% EDTA solution for 10 minutes at 37°C. Cell pellet was collected and resuspended in 5 mL of basal NSC medium. For sphere formation assay, ~1000 single cells from neurospheres at passage 4th were seeded into 24-well plate. Formation of new spheres was recorded.

**Immunocytochemistry**

To examine Nestin expression, passage 4th neurospheres were collected, fixed in 1 mL of 1X FCM fixation buffer at RT, 30 mins and ice-cold, 5 mins 1X FCM permeabilization buffer (Santa Cruz Biotechnology, USA). The sphere was incubated with 1st rabbit anti-mouse nestin antibody (1: 200 NS413, Sigma Aldrich, Singapore), then FITC-conjugated 2nd anti-Rb antibody (1:5000 ab6717, Abcam Singapore). Nuclei were stained with Hoescht 33342.

To examine GFAP expression, single cells from spheres were cultured in 2% FBS, EGF-free and bFGF-free basal NSC medium. Culture surface was covered with 50 μg/mL poly-L-Lysine to promote adhesion. After 10 days, spheres were fixed in FCM fixation buffer at RT for 5 minutes before being incubated with 1st rabbit anti-mouse GFAP antibody (1:100 ab16997, Abcam, Singapore) and rhodamine-conjugated 2nd anti-Rb antibody (4 μg/mL #31670 ThermoFisher Scientific, USA).

**Ginseng treatments**

For proliferation assay, 300μm-diameter neurospheres (n=10 spheres/each treatment) were used in ginseng treatment. The fraction was added to the basal medium with one of the concentrations 50, 100, 200 or 500 μg/mL). Basal NSC medium with or without 5 μg/mL nerve growth factor – NGF (Sigma Aldrich, St Louis, MO) was used as the negative and positive control, respectively. Diameters of the neurospheres were recorded every day for 5 days. For
After 5 days, treated neurospheres were subjected to cell cycle analysis and gene expression.

**Flow cytometry**

Neurospheres were dissociated by 0.025% trypsin, 0.02% EDTA for 10 minutes at 37°C. One million cells were incubated with 0.25 µg/mL PI (BD Biosciences, USA) at 37°C for 20-30 minutes. The DNA content was analyzed using the FACSCalibur flow cytometer BD Biosciences and CellQuest Pro software. To analyze the cell cycle phase, cells were fixed with FCM fixation buffer (RT, 30 minutes) and ice-cold FCM permeabilization buffer (5 minutes), treated with 550 U/mL RNase A (Thermo Fisher Scientific, USA) at 37°C in 30 minutes. The cells were stained with 50 µg/mL PI (BD Biosciences, USA) at 37°C, no-light for 20-30 minutes. The DNA content was analyzed by the FACSCalibur flow cytometer BD Biosciences and CellQuest Pro software.

**Quantitative RT-PCR**

Total neurosphere RNA was extracted using Easy-Blue Total RNA Extraction Kit (iNtRON Biotechnology, South Korea). Real-time RT-PCR analyses were performed using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent, USA). Expression of cell cycle (ki67, cycA1, cycD1, cycC) and NSC markers (map2, gfap, mbp, sox2) genes were evaluated using Mastercycler® Ep Realplex (Eppendorf, Germany). Levels of expression were analyzed using Livek-method (2^(-∆∆Ct}).

**Statistical analysis**

Data in this study was presented as mean ± SEM and analyzed by GraphPad Prism 6.0 software. Differences amongst treated groups were analyzed by two-way ANOVA followed by post-hoc Tukey’s multiple comparisons methods. Differences would be considered statistically significant when p-value ≤ 0.05.

**RESULTS**

**Spheroid bodies emerging from floating cells expressed neural stem cells markers**

Three days since seeding, round-shape clumps of cell were seen in the culture (Figure 1A,B). Sphere formation assay showed that cells when separated from the sphere could form new ones (Figure 1B,C). Cells inside spheres were Nestin-positive for neural stem/progenitor marker (Figure 2 and CD24-positive by flow cytometry analysis (Figure 3). As withdrawing EGF and bFGF as well as adding fetal bovine serum to the basal NSC medium, cells adhering upon the surface were GFAP-positive (Figure 4).

**High concentration of n-butanol was non-neurotrophic, not sustaining the structure of cultured neurospheres**

The n-butanol fraction 500 µg/mL was unable to maintain the integrity of cultured neurospheres (Figure 5), characterized with scattered cells and dark borders. However, ethanol and aqueous fractions at concentrations did not cause any significant neurosphere deformity. Low concentrations (50, 100, 200 µg/mL) of the n-butanol fraction seemed not toxic for the neurospheres.

**Panax vietnamensis ethanol fraction 200 µg/mL could maintain the growth rate of treated neurospheres**

At day 4, the basal NSC medium (control) sphere diameter reached the final enlargement of 17±4%. At day 4 n-butanol 200 µg/mL and aqueous fraction 200 or 500 µg/mL significantly increased the sphere diameter compared to the control: n-butanol – 30.7±4.23% (p<0.001), aqueous fraction 200 µg/mL – 23.78±7.99% (p<0.01), aqueous fraction 500 µg/mL – 22.98±7.99% (p<0.01). However, there was no significant difference between the control and ethanol fraction 200 µg/mL neurosphere. At day 5, ethanol fraction 200 µg/mL increased the sphere diameter by approx. 28±3% (p<0.001), and no noticeable deformity of treated spheres was seen (Figure 6). No difference was between the growth rate of basal NSC medium and ethanol/n-butanol fractions 50 or 100 µg/mL.

For the integrity in neurosphere structure, those treated with 200 µg/mL n-butanol fraction (Figure 7) or 500 µg/mL aqueous fraction (Figure 8) could not maintain the whole structure at the end of the experiment. These spheres were characterized with loose cells around the border, eventually adhering upon the surface. Interestingly, treated neurospheres had a high and stable rate of diameter increase in the first three days, and began to degrade afterward significantly.
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ – 3’)</th>
<th>Genes</th>
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| gapdh | F: AAGTTGTCATGGGATGACC  
        R: TCACCATTCCAGGAGC | NM_001289726.1 |
| ki-67 | F: GCAGGAAGCAACAGATGAGACC  
        R: GCTCAGGTGATACATGCGCTCCTGC | NM_001081117.2 |
| cycA1 | F: GTTTCCCAATGCTGGTGA  
         R: AACAAATCCCGTTGCTTCT | NM_001305221.1 |
| cycD1 | F: CCAGAGGGGATGAGAACA  
         R: ATGGAGGTTGGTTGAAAT | NM_007631.2 |
| cycC | F: CAGGACATGGGCCAGGAA  
      R: TCCGTCCTGATGCTATCCACTATC | NM_001290420.1 |
| map2 | F: GCCACTCTCTCAAGCTACTCT  
      R: CTGGACGTTCTTAGGGGTCTG | NM_001310634.1 |
| glfap | F: AACGGCATCACCATTCTCTGT  
       R: ACCTCACCATCCCGCATCT | NM_010277.3 |
| mbp  | F: CTATAATCGGCTCACAAGG  
      R: AGGCGGTTTATATTAGAAAGC | NM_001025258.2 |
| sox2 | F: AAGGGTTCTTGCTGTTTTT  
      R: AGACCAGAAAACGGTCTTG | NM_011443.4 |

F: Forward; R: Reverse

Figure 1: Neural stem cell culture. (A) A sphere forming in primary culture (B) New cell clumps in secondary culture (C) A secondary neurosphere forming from cells of primary neurospheres.

Figure 2: Nestin-positive neurosphere. (A) Bright-field (B) Merged: nestin – FITC, nucleus – Hoescht33342 (C) Superimposed.
Figure 3: CD24-positive population in cultured neurosphere (A) Cells isolated from cultured neurospheres; (B) Unlabelled; (C) CD24-positive cells in the population.

Figure 4: Glial fibrillary acidic protein (GFAP) expression in differentiation-induced neural stem cells (A) Bright-field (B) Intracellular expression of GFAP – rhodamine, nucleus – Hoescht 33342 (C) Superimposed image

Figure 5: Sphere integrity treated with n-butanol fraction 500 μg/mL (A) The sphere after treating for one day, and (B) degraded with its cells dispersed, lost its entire structure on day 2.
Table 2: Summary of the treated neurosphere condition

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Concentration (μg/mL)</th>
<th>Neurotrophic</th>
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<tbody>
<tr>
<td>n-butanol</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50 – 200</td>
<td>+</td>
</tr>
<tr>
<td>Aqueous</td>
<td>50 – 500</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50 – 500</td>
<td>+</td>
</tr>
</tbody>
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(−) non-neurotrophic; (+) maintain development until the final day

**Figure 6:** Diameter growth rate of cultured neurospheres the growth percentage is presented as mean±SEM (**p<0.1; #p<0.001).
Figure 7: Neurosphere integrity when treated with 200 μg/mL ethanol or n-butanol fraction after 5 days. Spheres treated with n-butanol fraction at 200 μg/mL lost their intact structure and cells began to detach from the sphere, adhering to the surface in shape of flatten or round cells around the sphere.

Ethanol fraction of Panax vietnamensis 200 μg/mL elevated G2/M-phase cells and cell cycle-related genes

For further analysis on the gene expression and cell cycle, 200 μg/mL was chosen as the only concentration of each ginseng fraction. The G2/M percentage of the ethanol fraction neurospheres was 28.73±0.44% (p<0.001) and aqueous fraction was 25.85±0.71% (p<0.01). For comparison, the basal NSC medium had 16.88±2.76% G2/M. The proportion of G0/G1 phase declined in all fraction-treated groups compared with that of basal NSC medium (p<0.0001); the most significant was that of n-butanol fraction-treated spheres (28.64±1.63%, p<0.0001). n-Butanol fraction-treated spheres had 51.2±0.93% cells in S phase (p<0.001), but not significantly increase the proportion of G2/M-phase cells (20.2±0.71%) (Figure 9 A).

Treating neurospheres with 200 μg/mL ethanol fraction significantly elevated the mRNA levels compared with those of the basal NSC medium: ki67 (4.60±6.48 fold-change), cycC (9.53±2.63 fold-change), cycD1 (22.47±8.18 fold-change), cycA1 (12.61±4.65 fold-change). However, there was a down-regulation in all surveyed genes compared with the basal NSC medium when treating spheres with the n-butanol or aqueous fraction (Figure 9 B).

Maintaining high level of sox2 and gfap expression as treating neurospheres with Panax vietnamensis ethanol fraction at 200 μg/mL

To evaluate the differentiation effect of the ginseng fractions, neurospheres were cultured in EGF- and bFGF-free media, with the ginseng fraction added for five days. In addition, NGF (5 μg/mL) was also added as the positive control in the differentiation assay. In this study, there was a high mRNA level of sox2 (71.25±27.24 fold-change) and gfap (73.55±47.14 fold-change) as treating spheres with 200 μg/mL ethanol fraction. These levels were significantly different compared with those treated with the n-butanol fraction (sox2: 4.62±4.72 fold-change, p<0.05; gfap: 0.85±1.02 fold-change, p<0.01) and aqueous fraction (sox2: 5.77±1.44 fold-change, p<0.05; gfap: 0.66±0.20, p<0.05). The map2 mRNA
level in ethanol fraction-treated neurospheres was up-regulated (4.605±3.33), but not statistically different from that in other groups. Interestingly, the mbp mRNA level of all treatment groups were down-regulated as compared with the negative control (Figure 10).

**DISCUSSION**

In this study, cultured neural stem cells could persistently generate secondary spheres through 4 passages, and strongly expressed nestin and CD24, markers for neural lineage. Neural stem/progenitor cells could differentiate to 3 distinct types in the neural lineage: neurons, astrocytes and oligodendrocytes. Cells from neurospheres could be induced to differentiate into the GFAP-positive astrocyte-like cells.
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Figure 10: Differentiation mRNA expression. Graph showing mean±SEM of 2−ΔΔCt of differentiation gene mRNAs in treatment groups normalized to the basal NSC medium control: sox2 – neural stemness, map2 – mature neuron, gfap – astrocyte, mbp – oligodendrocyte. Dotted line refers to the 1-fold change.

Further confirming the expression of GFAP protein, which was previously mentioned by mRNA expression in our previous study. Our results show that ginseng extract fractions significantly promoted the neurosphere growth. Normally, quiescent cells predominantly present in cultured neurospheres, which was confirmed by the high proportion of G0/G1 in those cultured with the basal NSC medium. When treating neurospheres with ginsenosides, it was shown that they promote the growth rate of neurospheres both in vitro and in vivo. In this study, the P. vietnamensis ethanol fraction particularly enhanced the proliferation of neural stem cells compared with other fractions. Interestingly, there was a similar pattern between ethanol fraction- and NGF-treated neurospheres: up-regulated mRNA levels of proliferating genes and high G2/M proportion. In the presence of EGF and bFGF, nerve-growth factor (NGF) increases the number of nestin+ cells and promotes the survival and proliferation of neural stem cells.

Ginsenosides were shown to enhance the expression of the neurotrophic receptor such as p75, p21, TrkA in Neuro-2a cells as well as elevate NGF and BDGF levels in cultured Schwann cells. This suggest that the ethanol fraction might have similar effects of NGF on proliferating neurospheres.

In the differentiation assay, there was also a similar pattern between ethanol fraction- and NGF-treated neurospheres. Interestingly, our results indicated up-regulation of cycD1 mRNA and decrease in G0/G1 population effect in the proliferation assay (shown above), which suggests neurogenesis inhibition while self-renewal promotion. This was correlated with the high sox2 mRNA level in the absence of EGF and bFGF coming from actively self-renewal cells. In addition, actively proliferating neurospheres would contain GFAP+ core due to being partly isolated from mitogens, correlating with the high mRNA level of gfap when eliminating EGF and bFGF from the medium. In this study, the ethanol fraction-treated neurospheres were more condensed than those with n-buthanol fraction indicating an increase in the size of individual cells rather than the cell number. This was consistent with a significantly high level of S-phase cells but low level of ki67 and cycC mRNA in n-butanol fraction-treated neurospheres. As treating neurospheres with the aqueous fraction, low cycC mRNA level and S-phase proportion suggest that treated cell poorly entered active stages. With the presence EGF and bFGF in culture media, it’s noteworthy that the ginseng n-buthanol or aqueous fraction might have inhibiting effect on the neural stem cell proliferation.

Previous studies on Panax vietnamensis extract already presented its new ginsenosides and other bioactive substances as well as its in vivo effects on the nervous system. Others already pointed out positive effects of Panax ginseng extract/ginsenosides on nervous system in vivo of increasing SOX2 expression and promoting hippocampal proliferation.
attenuating neural stem cell senescence, maintaining neural stem cell proliferation in lead poisoning. Because neural stem/progenitor cells still reside in the body, many questions concerning specific mechanisms of ginseng extract/ginsenosides still remain. Using an in vitro model of neurosphere, for the first time this study has provided new insights into proliferative and differentiative effects of the ginseng extract fractions, particularly the ethanol fraction on the neural stem cell. However, further experiments should focus into specific Panax vietnamensis ginsenosides to elucidate how the ginsenosides could promote or inhibit the neural stem cell proliferation/differentiation.

CONCLUSIONS

In this study, Panax vietnamensis extract fractions of at specific concentrations had a positive effect on the proliferation of cultured neural stem cells. The ethanol fraction at 200 μg/mL could significantly promote the growth rate while still sustained the integrity of treated spheres. Treated neurospheres had high levels of cell cycle mRNA expression, high proportion of the G2/M cells, as well as the percentage of G0/G1 significantly decreased. Moreover, the fraction might have similar effects as those of NGF on the differentiation of neural stem/progenitor cells. Further study should be done to elucidate the mechanism in which each ginsenoside has its effects on neural stem cells.

ABBREVIATIONS

bFGF: basic fibroblast growth factor
EGF: Epidermal growth factor
GEAP: Giall Fibrillary Acidic Protein
NGF: Nerve growth factor
NSC: Neural stem cell

COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

AUTHORS’ CONTRIBUTIONS

HQ Do and NH Truong carried out studies including gene-expression, flow cytometry, data analysis and manuscript composing. TT Lam, LT Nguyen, NHT Dinh and PTB Le isolated/cultured neural stem cells and tested gingseng fraction on neural spheres. LC Tran performed plant fractions for the experiment. NK Phan and PV Pham, who advised and orient the study, revised the manuscript, edited figures and checked the published data. All authors read and approved the final manuscript.

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