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Evaluation of effects of Lingzhi mushroom (*Ganoderma lucidum*) on neural stem cells isolated from embryonic mouse brain (*Mus musculus* var. *albino*)

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Abstract

Neural stem cells (NSCs) are employed as a promising tool for the replacement of damaged cells in several neurological disorders, including Parkinson's, Alzheimer's, Huntington's diseases and spinal cord injury thanks to their self-renewal and pluripotency. However, due to the limitation in site-specific migration of endogenous stem cells, exogenous NSC sources, of which growth can be triggered by extrinsic factors, are taken advantage of. This study aims to evaluate the role of *Ganoderma lucidum* extract on neural stem cell proliferation as well as determine the concentration having the best growth stimulating effects. NSCs were isolated from 13.5 – 15.5 day embryonic mouse brain (*Mus musculus* var. *albino*) and cultured in DMEM/F-12 serum-free medium modified with B27, N2, heparin, EGF and FGF at 37^{o}C, 5% CO_2. Characteristics of candidate cells were evaluated by neurosphere assay, differentiation assay as well as the expression of Nestin, CD133 and Sox-1 by immunocytochemistry, flow cytometry and RT-PCR techniques, respectively. Those NSCs were then cultured in 96-well plate with *Ganoderma lucidum* extract at 100μg/ml, 500μg/ml and 1000μg/ml for evaluating mitogenic effects of such mushroom. The results revealed that 72% of cell sample was successfully cultured, cells isolated from 13.5-15.5 days post coitum fetus showed a high proliferation rate, isolated cells formed neurospheres, expressed neural stem cell markers and differentiated into GFAP positive cells. *Ganoderma lucidum* extract at 500μg/ml showed the best effects on neural stem cell growth.

Keywords: *Ganoderma lucidum*, neurosphere assay, neural stem cell markers, neural-colony forming cell assay, neurosphere size.

1. Introduction

Neurodegenerative diseases, the quantity loss of functional neural cells in the brain, spinal cord and nerves due to apoptosis or necrosis, could affect body movement, awareness, respiration, emotion and memory. According to Stump (12), there were 1 billion patients suffering neurodegenerative diseases in 2007, with 50 million cases of epilepsy and 24 million cases of Alzheimer, resulting in 6.8 million cases of death annually. Thus, such diseases, with their dramatic growth, have been raising a great concern among the general public over the past 65 years. Among those, Alzheimer's disease and Parkinson's disease are the most popular neurological disorders in the world. These have been treated by medication or conventional surgery; however, many side-effects of the medicine were addressed. In the same context, surgery was reported as an inefficient therapy since patients had to undergo surgery for several times, but some symptoms still remained. Recent studies have discovered potential uses of stem cells in treating such disorders, especially Parkinson's disease for its enormous efficacy. These cells possess the capacity to self renew and differentiate into astrocytes, neurons and oligodendrocytes. Moreover, neural stem cell proliferation and differentiation could be triggered by appropriate extrinsic factors in *in vitro* conditions (9).

Ganoderma lucidum is a fungus widely used in Chinese, Japanese, Korean and Vietnamese medication for several years (7) for its immunomodulatory, antioxidant, antitumor, antihypertensive and tranquilizing activities. Triterpenes, polysaccharides and peptidoglycans are reported as the key components responsible for its important biological activities. Besides, Lingzhi-8 is documented as a mitogen-like protein that could promote cell division *in vitro*. Such mushroom extract was demonstrated to induce neuronal phenotype formation of pheochromocytoma cells and protect neurons from apoptosis due to NGF withdrawal (2). According to Zhu (15), Lingzhi extract could prevent the loss of dopaminergic neurons in the striatum.

2. Materials and Methods

2.1 Isolation and culture of embryonic neural stem cells

Embryonic neural stem cells were collected from mouse embryos (*Mus musculus* var. *albino*) at ages 13.5 – 15.5 days post coitum. In brief, mouse fetus was decontaminated in sterile phosphate-buffered saline (PBS) solution with 5% penicillin/streptomycin (Sigma-Aldrich). These fetuses were decapitated and rinsed in sterile PBS solution defined with 1% antibiotics prior to brain separation. This was followed by the mechanical dissection of the separated brains, and suspended cells were filtered through a 70μm cell strainer (BD Biosciences). Cells were collected by centrifugation (2500 RPM, 5 minutes) and resuspended in a serum-free medium containing DMEM/F-12 (Sigma-Aldrich), 30% glucose, 20ng/ml EGF (Sigma-Aldrich), 20ng/ml b-FGF (Sigma-Aldrich), 1μg/ml insulin (Sigma-Aldrich), 5μg/ml transferrin (Sigma-Aldrich), 1M HEPES buffer (Sigma-Aldrich), 2.5IU/ml heparin (Sigma-Aldrich), 1% gentamycin (Sigma-Aldrich), B27 and N2 growth supplement (Gibco). These cells were cultured in 6-well plates and incubated at 37°C, 5% CO_2. Cell passages were carried out after 2-3 days of culture.

2.2 Neurosphere assay

Spheres of passage 4 were collected by centrifugation (1500 RPM, 5 minutes), followed by the resuspension and dissociation in serum-free medium. These single cells were then plated at a density of 10^4cells/ml in 96-well plate at 37°C, 5% CO_2. Cell proliferation was observed and evaluated every 24 hours.

2.3 Reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR analysis, spheres of passage 4 were

collected by centrifugation (1500 RPM, 5 minutes) prior to RNA extraction. RNA was then isolated by using the easy-BLUE kit (iNtRON Biotechnology, Inc) according to the manufacturer's instructions, and RT-PCR was carried out employing One-step RT-PCR premix kit (iNtRON Biotechnology, Inc). Primer sequences (forward and reverse) were as follows: Sox1: 5'-CATCTCCAACT CTCAGGGCT-3', 5'-ACTTGACCAGAGATCCGAGG-3' (14); GAPDH: 5'-AAGTTGTCATGGATGACC-3', 5'-ATCACCATCTTCCAGGAGC-3' (3). Amplification procedure was set up as follows: 42°C for 60 minutes, 70°C for 5 minutes, 95°C for 10 minutes, followed by 30 cycles of annealing at 95°C for 15 minutes, 57°C and 51°C for 30 seconds, 72°C for 30 seconds and the extension at 72°C for 10 minutes.

2.4 Flow cytometry

Spheres for flow cytometry were harvested by centrifugation (1500 RPM, 5 minutes) and mechanically dissociated to a single-cell suspension in 0.25% Trypsin/EDTA solution. Such cells were incubated at 37°C for 2 minutes and collected by centrifugation (2500 RPM, 5 minutes). The pellet was suspended in FACS Fluid solution containing CD133 antibody (1:200; Sigma-Aldrich) labeled with FITC fluorescence (1:200; Santa Cruz Biotechnology, Inc). This mixture was incubated on a shaker at room temperature for 15 minutes and centrifuged to harvest cells. Cells were resuspended in FACS Fluid solution and cell sorting was performed on FACSCalibur flow cytometer (BD Biosciences).

2.5 Immunocytochemistry

Spheres were pre-incubated in 1% BSA solution at room temperature for 10 minutes, followed by a rinse in PBS solution and centrifugation (1500 RPM, 5 minutes). The pellet was then resuspended in FCM fixation buffer (Santa Cruz Biotechnology, Inc) and incubated for 30 minutes at room temperature on a rotator. Spheres were washed and centrifuged (1500 RPM, 5 minutes) before being permeabilized in FCM permeabilization buffer (Santa Cruz Biotechnology, Inc) for 5 minutes. The buffer was removed by centrifugation (1500 RPM, 5 minutes) and spheres were resuspended in a solution containing anti-Nestin antibody (1:200; Sigma-Aldrich) labeled with FITC (1:200; Santa Cruz Biotechnology, Inc) and Hoechst 33342 (1:200; Sigma-Aldrich) at room temperature for 60 minutes. Finally, spheres were collected by centrifugation, resuspended in PBS and plated in 24-well plate. Images were taken with an inverted fluorescent microscope with AxioVision software and processed with Adobe Photoshop CS3 software.

2.6 Differentiation to astrocytes and GFAP expression of differentiated cells

Spheres were first collected by centrifugation (1500 RPM, 5 minutes) and resuspended in a medium containing DMEM/F-12 (Sigma-Aldrich), heparin (2.5IU/ml; Sigma-Aldrich), insulin (1μg/ml; Sigma-Aldrich), transferrin (5μg/ml; Sigma-Aldrich), 1% gentamycin (Sigma-Aldrich), B27 and N2 (Gibco). Cells were cultured at 37°C, 5% CO_2 for 10 days, then differentiation medium was removed and cells were fixed by FCM fixation buffer, followed by the incubation for 30 minutes at room temperature on a rotator. After fixation, cells were stained with anti-GFAP antibody (1:200; Sigma-Aldrich) labeled with Rhodamine (1:200; Santa Cruz Biotechnology, Inc) and Hoechst 33342 (1:200; Sigma-Aldrich) for 60 minutes at room temperature in dark condition. Stained cells were observed under inverted fluorescent microscope.

2.7 Evaluation of the effects of *Ganoderma lucidum* on cell proliferation

Neurospheres (100-250μm) were plated in 96-well plate (1-2 sphere(s)/well) and cultured in NSC medium containing *Ganoderma lucidum* extract at different concentration: 0μg/ml (Control), 100μg/ml, 500μg/ml and 1000μg/ml (37°C, 5% CO_2). Each treatment was repeated 3 times. The experiment with 1 factor was arranged randomly. Changes in neurosphere sizes were recorded every 24 hours for 4 days.

2.8 Statistical analysis

Changes in neurosphere sizes were presented as Mean ± SD and differences among means were assessed by the Student t-test. Differences were considered significant when the p-value was less than 0.05.

3. Results

3.1 Isolation and culture of candidate NSCs from embryonic mouse brain

10 out of 14 samples were successfully cultured and candidate NSCs isolated from the 13.5 – 15.5 days post coitum embryonic brains show a high rate of proliferation and sphere formation. Initially, many types of cell were observed, including erythrocytes, fibroblasts and candidate NSCs in the culture samples. However, these non-neural cells were eliminated after passages. Isolated candidate NSCs had round-shape morphology, bright nucleus and sized between 8-12μm (Figure 1A), which was also described by Schumacher (10). These cells grew in neural stem cell culture medium and formed small clusters within 24 hours of culture. After 3-4 days, some dead cells, which could not grow in this specific medium aggregated into opaque clumps, and several clusters of candidate cells with the diameters around 50μm formed (Figure 1B). 100-150 μm spheres were observed after 5-7 days and reached the size of approximately 250μm in day 10 of culture (Figure 1C). Similar results were also demonstrated by Ge et al. (5).

Passages were carried out after 2-3 days of primary culture to remove toxic metabolites, dead cells, provide nutrients and space for cell growth. When being mechanically dissociated, opaque clumps were removed and clusters of 3-4 cells could be observed. These clusters formed more small spheres within 24 hours and achieved a clearer round-shape in 3-4 days. They continuously increased in size and obtained a distinct spherical and compact structure after passage 3 or 4. After passage 7 or 8, cells in spheres died, leading to the loss of this structure.

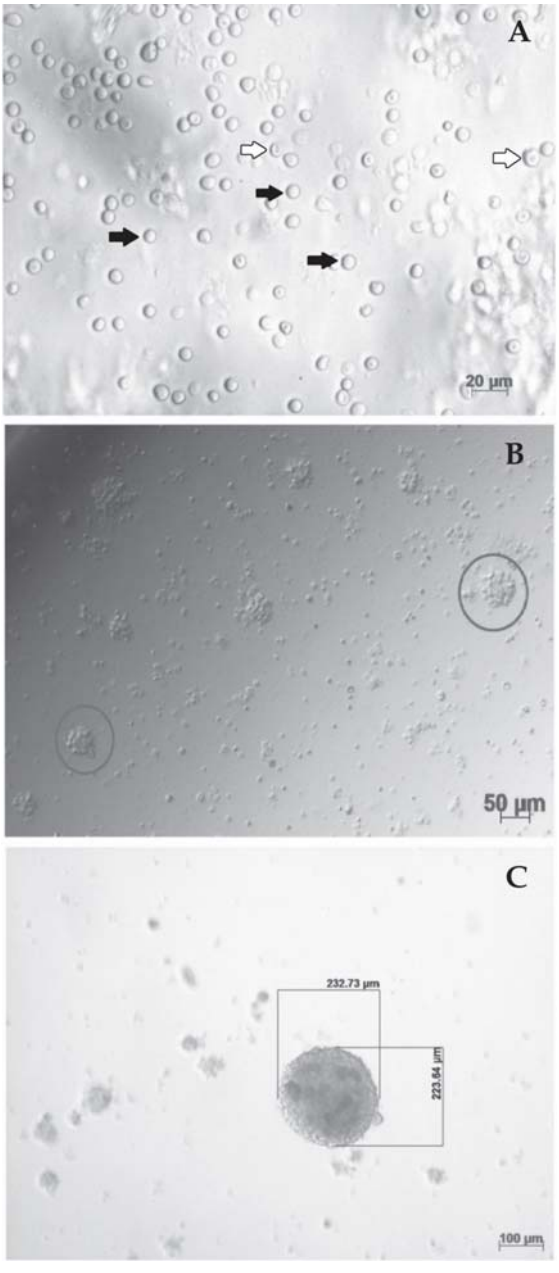

Figure 1. Sphere formation in primary culture. (A) Candidate cells with round shape (black arrows) and erythrocytes (white arrows). (B) Small clusters of cells formed after 3 days of culture. (C) Spheres with compact structure formed after 11 days of culture.

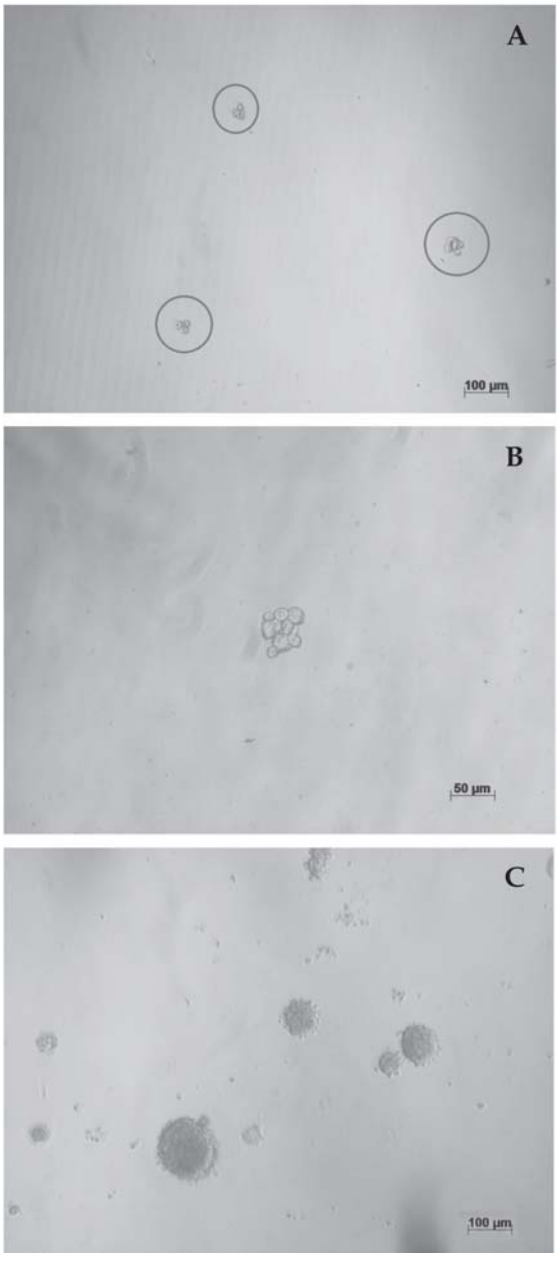

Figure 2. Sphere formation in neurosphere assay. (A) Clusters of 3-4 cells after dissociation. (B) A cluster of cells after 24 hours. (C) Spheres with distinct structure on day 7 of culture.

3.2 Neurosphere assay

Spheres of passage 4 were mechanically dissociated and cultured in 24-well plate. After 24 hours, small clusters formed (Figure 2B) and spheres with the diameter around 50µm long were obtained in the next 2-3 days. These neurospheres became more compact and had the diameters ranging from 100-200µm after 5-7 days (Figure 2C) and 250-400µm after passage 2-3.

3.3 Expression of NSC markers

To detect the expression of *Sox1*, total RNA extract of candidate neural stem cells was analyzed by RT-PCR. The 200bp band observed on the agarose gel confirmed the expression of *Sox1* in these neural stem cells (Figure 3). The flow cytometry histogram also revealed that 59.91% of candidate cell was CD133 positive (Figure 5). Moreover, stained spheres showed green fluorescence of FITC and blue fluorescence of Hoechst 33342 (Figure 4), indicating that they integrated with labeled anti-Nestin antibody, thus these were Nestin positive cells; however, less cells located at outer layers expressed this protein.

3.4 GFAP expression of differentiated cells

Cells were mechanically dissociated and cultured in differentiation medium containing 10% FBS without EGF and FGF. These cells began to adhere to the culture plate within 48 hours and underwent dendrite outgrowth. After 10 days of non-orientable differentiation, mature neural cells were present in the medium.

Figure 6 showed that candidate cells emitted red fluorescence of Rhodamine and blue fluorescence of Hoechst 33342, implying these cells were GFAP positive.

Table 1. The increase in neurosphere diameters after 72 hours of culture

Treatments	Percentage of diameter incrase
100 µg/ml	10.77 ± 0.61^{bc}
500 µg/ml	16.39 ± 0.78^{a}
1000 µg/ml	10.34 ± 1.18^{bc}
Control	10.33 ± 0.95^{c}

* Values were presented as Mean ± SD with 95% of confidence. Similar letters revealed that compared values were not significantly different ($p < 0.05$).

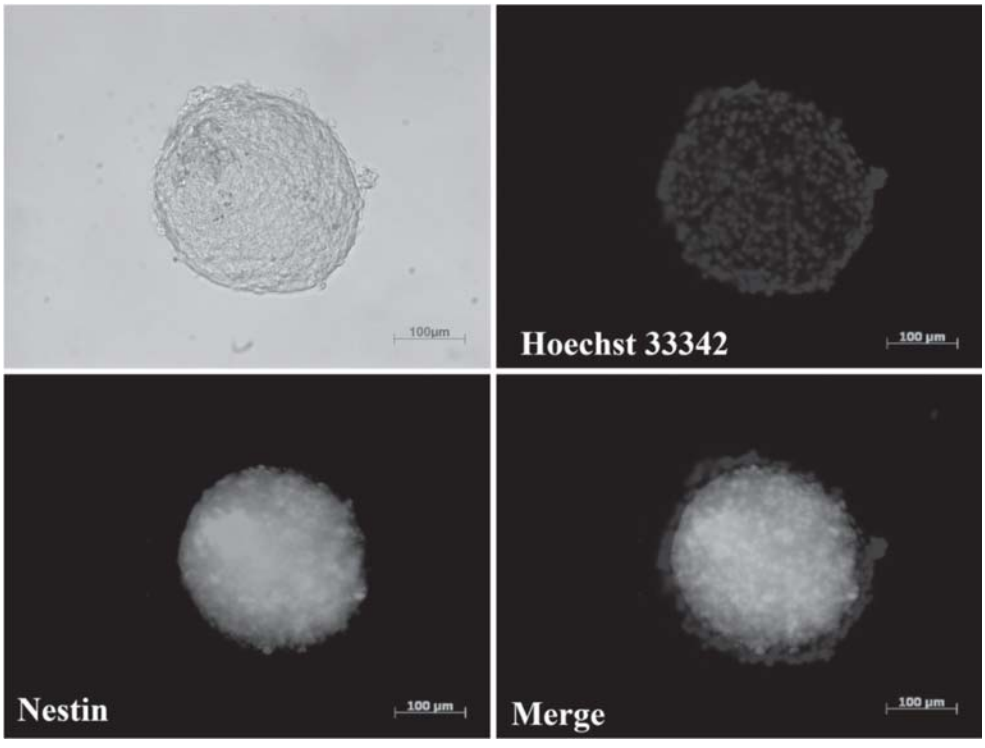

Figure 3. Nestin expression of candidate neural stem cells cultured in DMEM/F-12 serum-free medium modified with B27, N2, heparin, EGF and FGF.

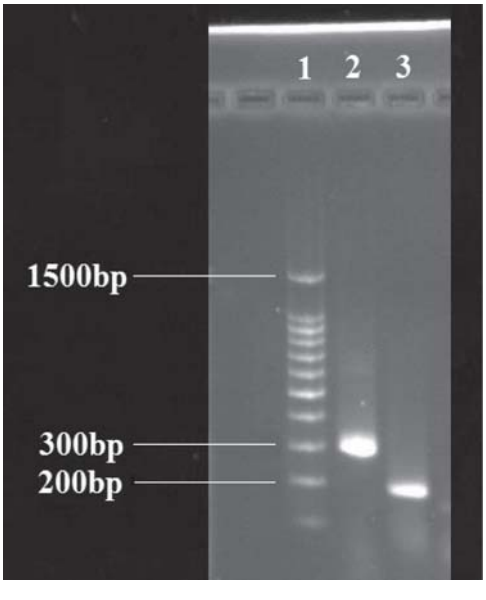

Figure 4. Electrophoresis of RT-PCR products. 1: Ladder, 2: *GAPDH*, 3: *Sox1*.

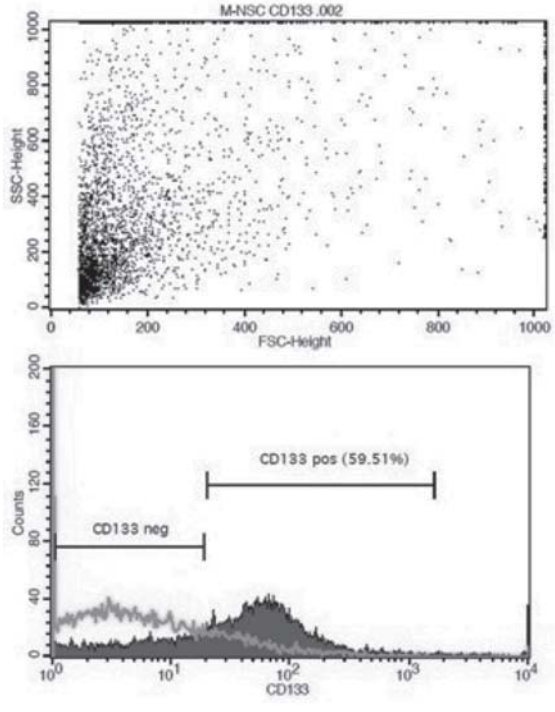

Figure 5. Histograms revealed CD133-positive cell population.

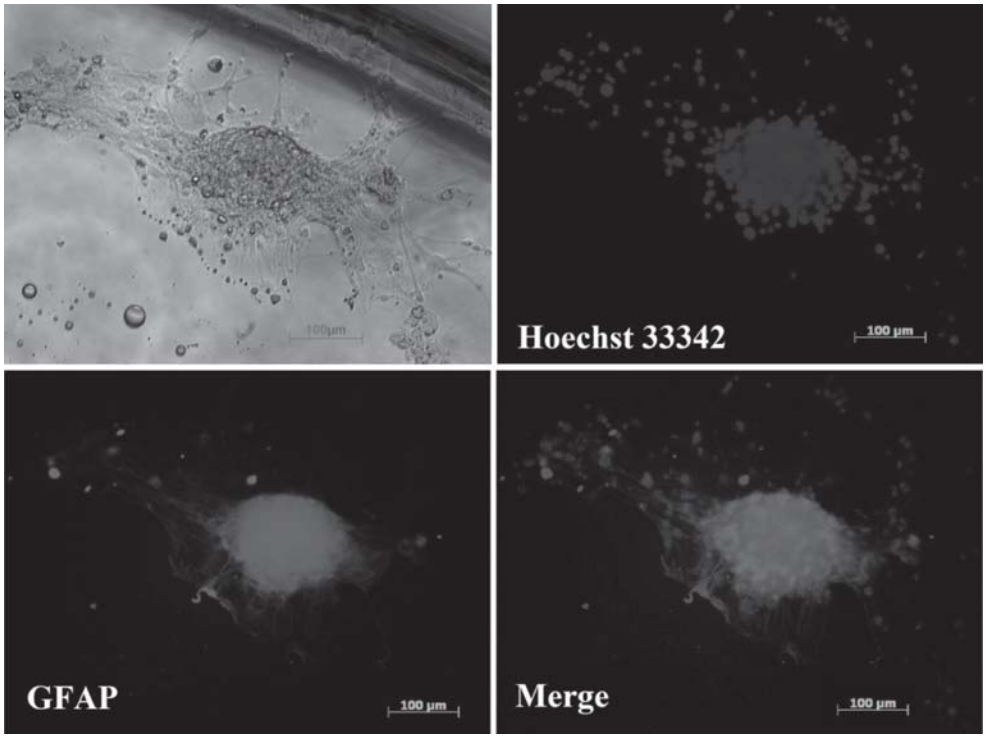

Figure 6. Fluorescent photomicrographs represented CD133-positive cell population GFAP positive cells after 10 days of non-orientable differentiation.

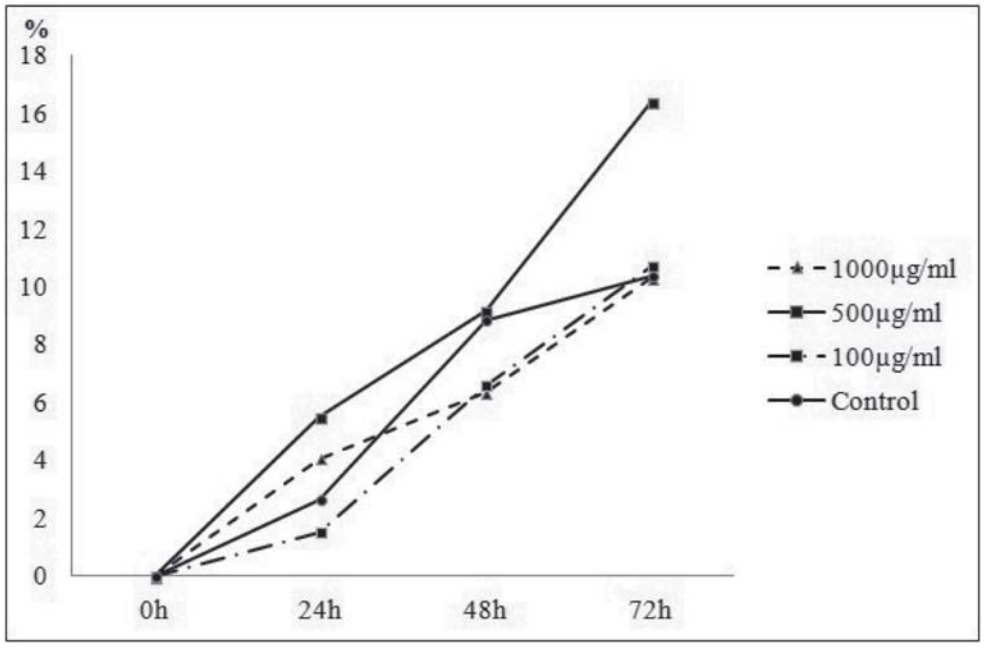

Figure 7. Changes in spheres diameters at different culture time.

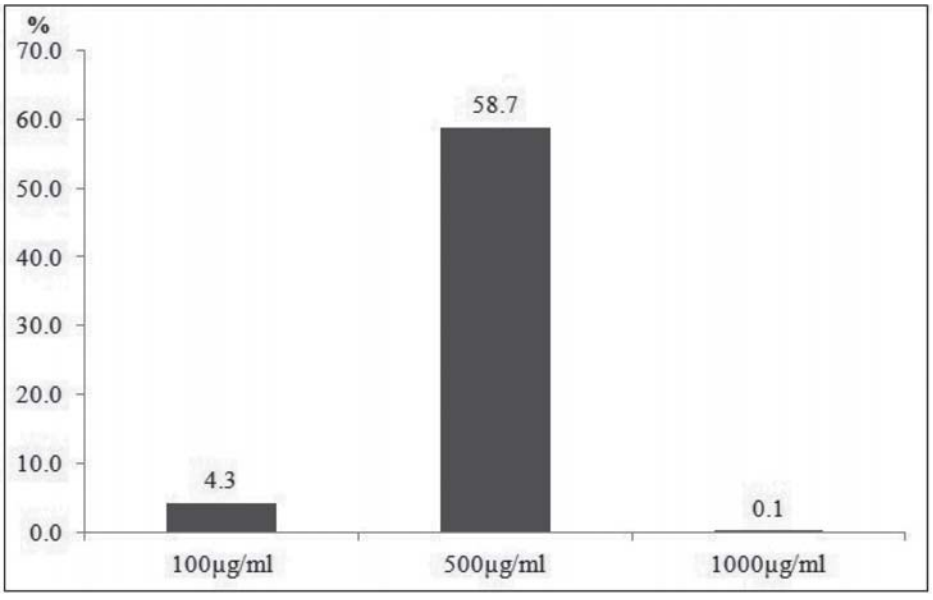

Figure 8. Size change of spheres in medium containing *G.lucidum* extract at different concentrations compared to the Control treatment after 72 hours of culture.

3.5 Mitogenic effects of *Ganoderma lucidum* on neural stem cells

From Table 1 and Figure 7, *Ganoderma lucidum* extract at the concentration 500µg/ml showed the best effect on cell growth in the first 72 hours, with 16.39%. In comparison to the Control, stimulating effect of the 500µg/ml treatment was 58.7% higher (Figure 8), and these treatments were significantly different. However, such comparative figures for the 100µg/ml and 1000µg/ml treatments were very low (4.3% and 0.1%, respectively). In addition, the highest and the lowest concentrations were not significantly different from the treatment without *Ganoderma lucidum*.

After 96 hours of culture, the bright color of spheres turned dark, adhered to the culture surface and began to differentiate. Some dead cells which dissociated from those spheres could also be observed.

4. Discussion

When studying the proliferation of cell in *in vitro* conditions, besides the number of generated neurospheres, neurosphere size should be considered since it correlates with the increased number of cells inside the aggregates. In fact, the larger the neurosphere diameter the higher number of cells inside it, and the lack of proliferative capacity prevents cells to generate clusters (4). Thus, in this study, neurosphere size was employed as a quantitative parameter for cell proliferation.

Ganoderma lucidum polysaccharides were demonstrated to enhance DNA synthesis, resulting in the increase in cell division (1). Hence, cells cultured in medium modified with such extract performed rapid proliferation. Moreover, Lingzhi-8 in *Ganoderma lucidum* was a mitogen-like protein, which also promoted mitosis and cell growth. Previous studies (11, 13) also demonstrated stimulating effects of *Ganoderma lucidum* on cell proliferation.

However, at higher concentration, ethanol remaining in the extract during fractionation could be harmful and toxic to cells. Some polysaccharide components, on the other hand, were proved to show toxicity on cells isolated from young patients undergoing chemotherapy (6). As a consequence, these substances could inhibit cell growth

and proliferation. At low concentration, growth stimulation elements in such extract could not also enhance the proliferation efficiently.

5. References

(1) About Ganoderma [Internet]. 2013 [cited 2013 Jul 3]. Available from: http://www.superganoderma.com/About_GanodermaGanoderma_Lucidum_Key_Effects/

(2) Cheung, WM, Hui WS, Chu PW, Chiu SW, Ip NY. Ganoderma extract activates MAP kinases and induces the neuronal differentiation of rat pheochromocytoma PC12 cells. FEBS Lett. 2000 Dec 15; 486:291-296.

(3) Diem TH. Modeling and treating interstitial hepatitis on *Mus musculus* var. *albino* by stem cell therapy [MSc thesis]. Ho Chi Minh University of Science; 2010.

(4) Fern!!ndez M, Paradisi M, Giardino L, Calz! L. To know neural stem properties from diseased brains: a critical step for brain repair. In: Grier EV, editor. Neural stem cell research. New York: Nova Science Publishers, Inc; 2006. p. 79-80.

(5) Ge D, Song K, Guan S, Dai M, Ma S, Liu T, Cui Z. Effect of the neurosphere size on the viability and metabolism of neural stem/progenitor cells. African Journal of Biotechnology. 2012; 11(17):3976-3985. doi: 10.5897/AJB11.3324.

(6) Gill SK, Rieder MJ. Toxicity of a traditional Chinese medicine, *Ganoderma lucidum*, in children with cancer. Can J Clin Pharmacol. 2008 Jul 4; 15(2):e275-e285.

(7) Kleinwatcher P, Anh N, Kiet TT, Schlegel B, Dahse HM, Hartl A, Grafe U. Colossolactones, new triterpenoid metabolites from a Vietnamese mushroom *Ganoderma colossum*. J. Nat. Prod. 2001; 64: 236-239. doi: 10.1021/np000437k.

(8) Quang DN, Nga TT, Tham LX. Chemical composition of Vietnamese black lingzhi *Amauroderma subbresinosum* Murr. Research Journal of Phytochemistry. 2011. doi:10.3923/rjphyto.2011.

(9) Reimers D, Gonzalo-Gobernado R, Herranz AS, Osuna C, Asensio MJ, Baena S, Rod"guez M, Baz!!n E. Driving neural stem cells towards a desired phenotype. Current Stem Cell Research & Therapy. 2008 Dec; 3(4):247-253.

(10) Schumacher A. Calculation of cell densities and cell layers in the outer shell of staurosporine induced "ovoid"-like neurospheres. Res Telegraph. 2003;1(1):1-7.

(11) Sliva D. *Ganoderma lucidum* (Reishi) in cancer treatment. Intergrative cancer therapies. 2003; 2(4):358-364. doi: 10.1177/1534735403259066.

(12) Stump E. WHO report: millions have neurological disorders worldwide. Neurology Today. 2007; 7(7):25. doi: 10.1097/01.NT.0000269134.28964.7e.

(13) Sun LX, Lin ZB, Li XJ, Li M, Lu J, Duan XS, Ge ZH, Song YX, Xing EH, Li WD. Promoting effects of *Ganoderma lucidum* polysaccharides on B16F10 cells activate lymphocytes. Basic & Clinical Pharmacology & Toxicology. 2010;108:149-154. doi: 10.1111/j.1742-7843.2010.00632.x.

(14) Tarantino C, Paolella G, Minopoli G, Pastore L, Parisi S, Tusso T. miRNA 34a, 100, and 137 modulate differentiation of mouse embryonic stem cells. The FASEB Journal. 2010; 24(9):3255-3263. doi: 10.1096/fj.09-152207.

(15) Zhu WW, Liu ZL, Xu HW, Chu WZ, Ye QY, Xie AM, Chen L, Li JR. Effect of the oil from Ganoderma lucidum spores on pathological changes in the substantia nigra and behaviors of MPTP-treated mice. Academic journal of the first medical college of PLA. 2005 Jun; 25(6):667-671.