Original Article

Combined effects of lithium chloride and butylated hydroxyanisole on neuronal differentiation of Wharton's jelly mesenchymal stem cells

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Abstract

Introduction: The potent neuron inducers which promote cell differentiation are necessary for the achievement of stem cell-base therapies. The chemical that widely used as neuron inducer is butylated hydroxyanisole (BHA). However, it's induced transient neuron differentiation. The previous studies showed that Wnt signaling pathway played an important role for development of neuron. The objective of this study was to evaluate the potential of lithium chloride (LiCl), the potent Wnt signaling pathway activator and BHA on neuronal differentiation from Wharton's Jelly-derived mesenchymal stem cells (WJMSCs).

Method: WJMSCs were cultured in media containing 1) 200 µM BHA 2) 5 mM LiCl and 3) the combination of 200 µM BHA and 5 mM LiCl. The 3 culture medium were used to induce neuronal differentiation in WJMSCs for 7 days. The morphological changes were assessed using hematoxylin staining. Cell differentiation was monitored by analysis of 3 neural gene expression, *nestin*, β-*tubulin* III and *neurogenin*, using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique.

Results: The two culture media, BHA or BHA combined with LiCl, induced the MSCs differentiate into neural-like cell phenotype. The RT-PCR results demonstrated the increased expression of all neural marker genes in BHA-treated cells. As well as, the expression of nestin and β-*tubulin III* were increased in LiCl-treated cells or LiCl combined with BHA while *neurogenin* expression was decreased.

Discussion and conclusion: Our findings provide basic information for neuronal differentiation from MSCs by BHA or LiCl. However, the mechanism of neural differentiation control by the inducers should be explored in further study.

Key words: Wharton's Jelly-derived mesenchymal stem cells, Neuron, LiCl, Butylated hydroxyanisole

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Introduction

The limited ability of neuron for self-repair and renewal has led to the investigation of alternative treatment options for neurological disorder as well as Alzheimer's disease (AD). The most promising approaches to neural repair and regeneration are the use of stem cell therapy. Mesenchymal stem cells (MSCs) are capable of differentiating into many cell types such as adipocytes,[1] osteocytes,[1, 2] chondrocytes[2-4] as well as neurons.[5-8] The MSCs from Wharton's Jelly (WJMSCs) have significant advantages over other cell source. These cells hold desirable characteristics such as rapidly available donor pool, painless, noninvasive collection procedure and autologous transplantation is possible for these cell types, thus avoiding the immune response.

Many reports showed that under chemical differentiation medium MSCs were induced morphology and expressed protein markers which were specific to neuron.[7, 9-12] The chemical that widely used as neuron inducer is butylated hydroxyanisole (BHA). However, it induces transient neuron differentiation and difficult to gain functional neuron after treatment of this chemical. Therefore, we attempted to introduce a new chemical to promote neural induction from WJMSCs. In this study, we were interested in LiCl because the LiCl has potential for Wnt signaling pathway activator.[13, 14] In addition, many previous studies showed that Wnt signaling pathway played an important role for development of the mammalian brain such as hippocampus, cotex as well as ventral mesencephalon.[15, 16] In contrast, lack of Wnt proteins led to retardation of the midbrain and rostral metencephalon.[17]

Therefore, we proposed to induce neuron from WJMSCs by using LiCl as a neuron inducer compared to BHA. The combination of the two chemicals was also performed to examine the neuronal differentiation. The cell morphology changes in differentiating media were assessed by using hematoxylin staining. Moreover, neural genes expression was performed by using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Flow diagram represented the methodology for neuronal induction by LiCl shown in Figure 1.

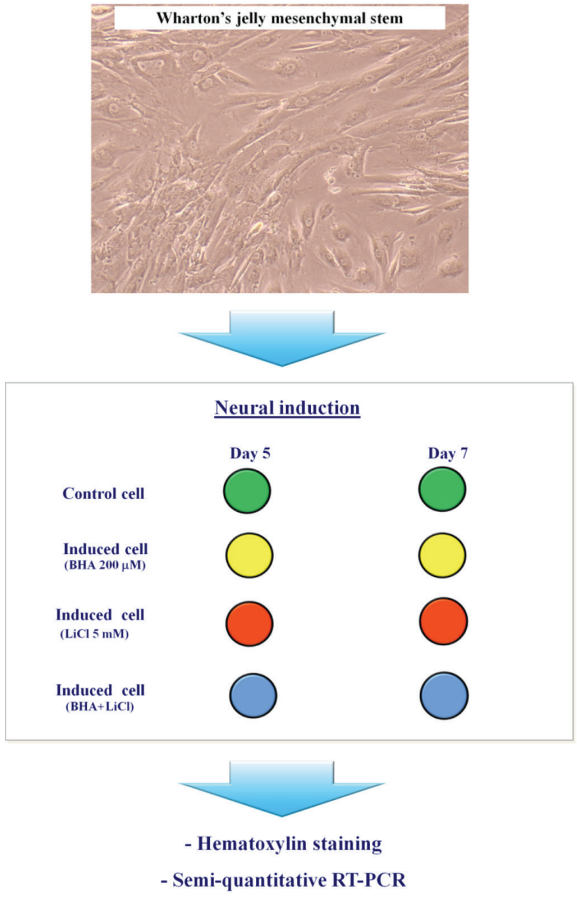

Figure 1 Flow diagram represented the methodology for neuronal induction

Method

WJMSCs culture

Human WJMSCs cell line, WJMSCs-SUT was kindly provided by Dr. Wilairat Leeanansaksiri (Suranaree University of Technology, Thailand). This cell line expresses cell surface markers: CD29+, CD44+, CD90+, CD34-, and CD45- and contains capacity of differentiation into osteocytes, chondrocytes, and adipocytes as standard characteristics of mesenchymal stem cells.[18] WJMSCs used as undifferentiated cells for neuron culture. Stem cells were incubator at 37°C with 5% CO_2 on Dulbecco's modified Eagle's medium with a low glucose concentration (DMEM–LG; Gibco, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) and 10% fetal bovine serum (FBS) (HyClone, Cramlington, UK). Medium was changed every 3 days. When the cells were 80% confluence, subculturing was performed using 0.25% trypsin-ethylene diamine tetra-acetic acetate (EDTA) (Gibco). WJMSCs passage 3 was used in the study.

Neural induction

Neural induction was performed as described previously by Woodbury et al., 2000.[7] In brief, the WJMSCs-SUT were seeded in the 24-well laminin-coated plate with DMEM-LG at a density of 1×10^4 cells/ml. Then, the culture media were replaced with preinduction media containing DMEM-LG, 20% FBS and 1mM β-mercaptoethanol (Sigma-Aldrich, USA). Twenty-four hours later, DMEM was removed and replaced with neural induction medium consisting of 2% dimethyl sulfoxide (DMSO), 200 µM butylated hydroxyanisole (BHA, Sigma-Aldrich), 25 mM KCl, 2 mM valproic acid (Sigma-Aldrich), 10 mM forskolin (Sigma-Aldrich) for 7 days. The other two culture media; prepared by adding 5 mM LiCl and 5 mM LiCl in combination with 200 µM BHA. In the presence of 5 mM LiCl, cell viability of stem cells was 100%.[19, 20] Non-stimulated control cells were cultured without the neural induction medium. Cells were collected on day 5th and day 7th for morphological changes observation, hematoxylin staining and gene expression profiles in order to examine the induction of WJMSCs to neuron.

Hematoxylin staining

The morphological changes were assessed using hematoxylin staining. After induced MSCs to neural cells for 7 days, the cells were washed three times with phosphate buffered saline (PBS), aspirated the PBS and fixed cells with neutral buffer formalin at room temperature for 20 min. Formalin was removed. The cells were washed twice with PBS. Then hematoxylin was added for 5 min and washed the MSCs again with PBS for 3 - 5 times. The cells were observed under an inverted phase contrast microscope for determine the neuronal morphology.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction

Total RNA was prepared from cell control and induced cells at day 5th and day 7th using RNeasy mini kit (Qiagen, Germany) based on the manufacturer's instructions. Samples were mixed with 70% ethanol. Then the mixture was applied to RNeasy spin column in a 2 ml collection tube for adsorption of RNA to the membrane and centrifuged for 15 sec at 10,000 rpm. The flow-through was discarded. The spin column was washed with 700 µl of buffer RW1 and centrifuged for 15 sec at 10,000 rpm. The spin column was washed again with 500 µl of buffer RPE and centrifuged at 10,000 rpm for 15 sec. The RNeasy silica-gel membrane was repeatedly washed with 500 µl of buffer RPE and centrifuged for 2 min at 10,000 rpm. The spin column was transferred into the 1.5 ml collection tube to elute the RNA. RNase-free water (30 - 50 µl) was pipetted directly onto the silica-gel membrane and centrifuged 10,000 rpm for 1 min. The purity and concentration of the extracted RNA were then checked by ultraviolet spectrophotometer at 260 and 280 nm.

cDNA synthesis

RNA was converted to cDNA by reverse transcription using a SuperScript III reverse transcriptase kit (Qiagen, Germany) based on the manufacturer's instructions. The reaction was set up on ice to avoid premature cDNA synthesis and to minimize the risk of RNA degradation. The reverse transcription reaction contained 1 µg of total RNA, 2.5 µM oligo (dT) 20 mers, 0.5 mM dNTP mixture. The RNA solution was heated at 65°C for 5 min. The mixture was added with 1X first-strand buffer, 0.1 M DTT, 40 units of RNase inhibitor and 200 units of SuperScript III reverse transcriptase. The RNA was incubated at 50°C for 60 min. Reverse transcriptase was then inactivated by incubating the reaction for 15 min at 70°C.

Semi quantitative RT-PCR

Cell differentiation was monitored by analysis of neural genes expression; *neurogenin 1 (Ngn1), nestin* and β-*tubulin III* using semi-quantitative RT-PCR. *GAPDH* was used as validated genes in this study. List of the primers used in this study were shown in Table 1 The amplification was carried out in a Thermal Cycler (Corbett Life Science, Australia). The PCR condition was optimized by varying annealing temperatures and concentrations of $MgCl_2$, primers and dNTPs. The reaction mixture was incubated initially at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 54 - 60°C for 40 sec and extension at 72°C for 60 sec. The amplified product was separated by 2% agarose gel electrophoresis. Quantitative analysis of the PCR results was done by GelAnalyzer 2010a (www.GelAnalyzer.com). Relative expression levels for the lineage specific genes were calculated by normalized neuronal gene expression to that of housekeeping gene, *GAPDH*.3

Table 1 List of the primers used for RT-PCR

Primer	Sequences	Size (bp)
Ngn1	Forward: 5' - AGT GAC CTA TCC GGC TTC CT - 3'	
	Reverse: 5' - TCA AGT TGT GCA TGC GGT TG - 3'	268
Nestin	Forward: 5' AAA GAG GGT TCA GGG CTT GG - 3'	
	Reverse: 5' CAA GGT GAA GGG GCA TCA CT - 3'	216
β-*tubulin III*	Forward: 5' - ACC TAC TGC ATC GAC AAC GA - 3'	
	Reverse: 5' - ACC TCC TTC ATG GAC ATG CG - 3'	383
GAPDH	Forward: 5' - GAG AAG GCT GGG GCT CAT TT - 3'	
	Reverse: 5' - AGT GAT GGC ATG GAC TGT GG - 3'	231

Results

Long term neural differentiation was performed to evaluate the differentiation of WJMSCs to neuronal lineage using BHA. As shown in Figure 2A-B, control cells predominately consisted with large flat spindle shaped cells which were regular shape of WJMSCs. After 5 days of MSCs induction by 200 µM BHA (Figure 2C), almost stem cells had small sized retracted their cytoplasm and emitted cellular protrusions. The neuronal-like phenotype were obviously observed after increasing culture period to 7 days (Figure 2D). However, some population of BHA-treated MSCs on the day 5th of induction retained adherence fibroblast-like cell morphology as shown in Figure 2C.

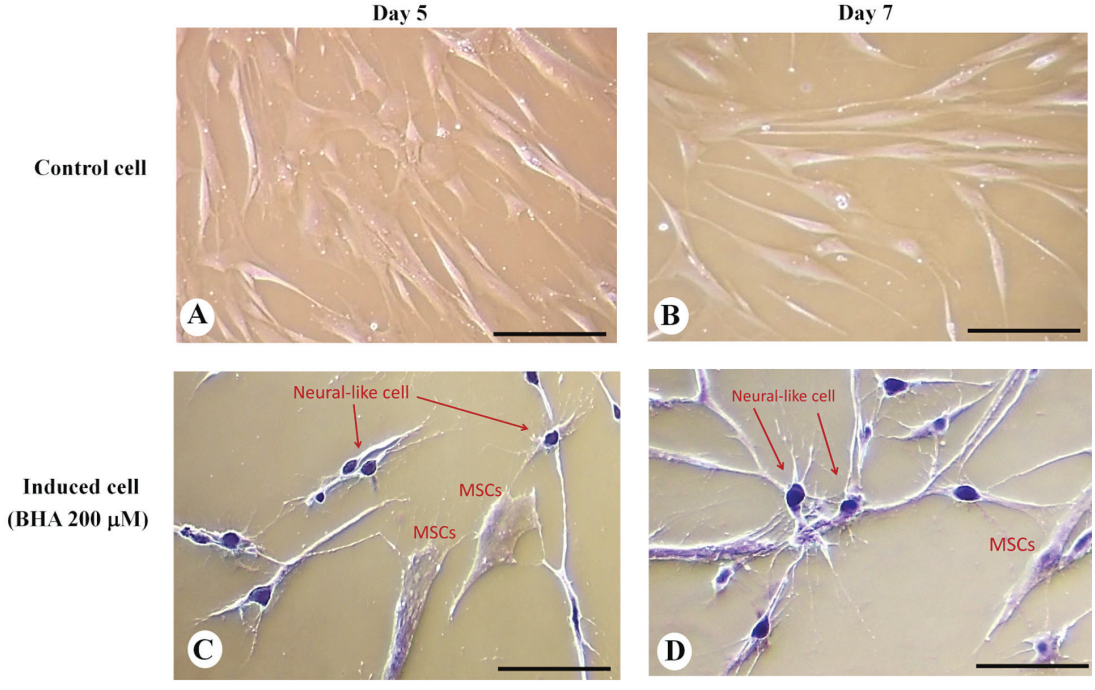

Figure 2 Hematoxylin staining demonstrated neural inductions by BHA. Undifferentiated WJMSCs retained adherence fibroblast-like cell (A, B). BHA-treated MSCs for 5 days (C) and 7 days, neuronal-like phenotype was observed (D). Most cells retracted their cytoplasm, forming spherical cell body and emitted cellular protrusions. Scale bar = 500 µm

Semi-quantitative RT-PCR was performed to detect the expression of neural genes in order to further analysis the BHA induction neuronal from WJMSCs (Figure 3). At indicated time-point of induction, the expression of *ngn1*, *nestin* and β-*tubulin III* were increased. These findings suggested that the WJMSCs might have potential to differentiate into neuron-like cells under different content BHA medium.

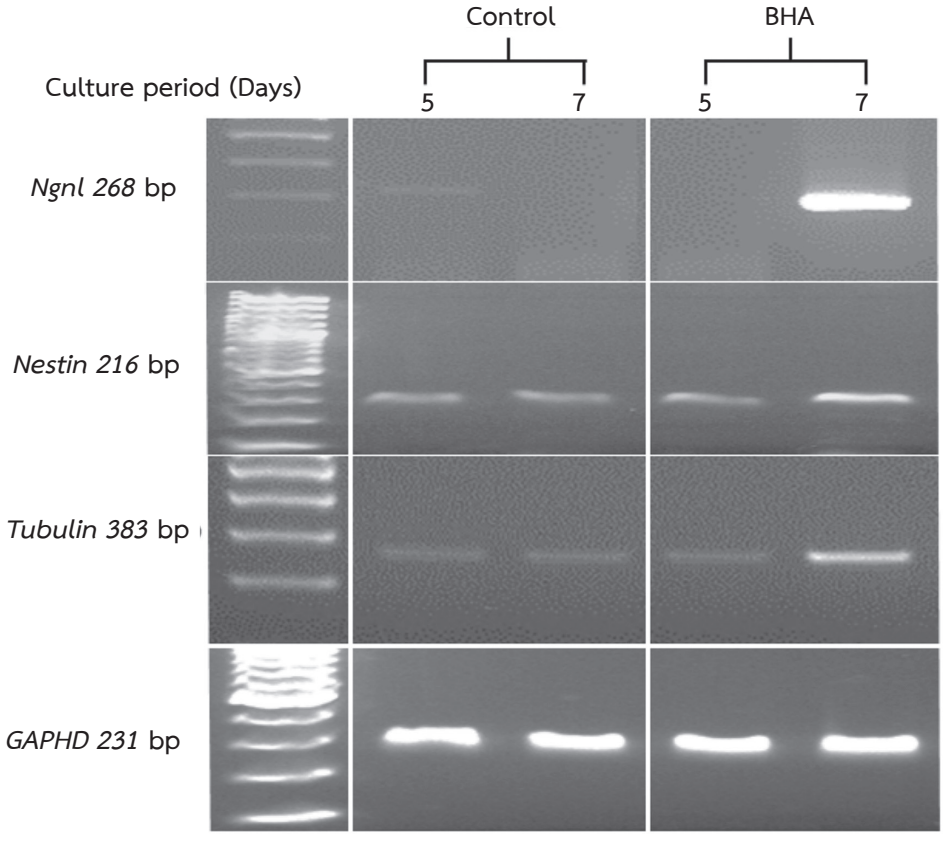

Figure 3 The results of semi quantitative RT-PCR at day 5th and 7th of induction showed expression of all neural marker genes was increased in BHA-treated WJMSCs compared with control cells

To study the effect of LiCl in neural induction, WJMSCs were cultured in two different media including LiCl and BHA combined with LiCl. As shown in Figure 4A-B, control cells were regular shape of WJMSCs. After chemical stimulation for 5 and 7 days a very small number of MSCs were change their phenotype like neuron when treated cells with BHA in combination of LiCl as demonstrated in Figure 4E-F. In contrast, LiCl-treated MSCs could not change the morphology of stem cells (Figure 4C-D).

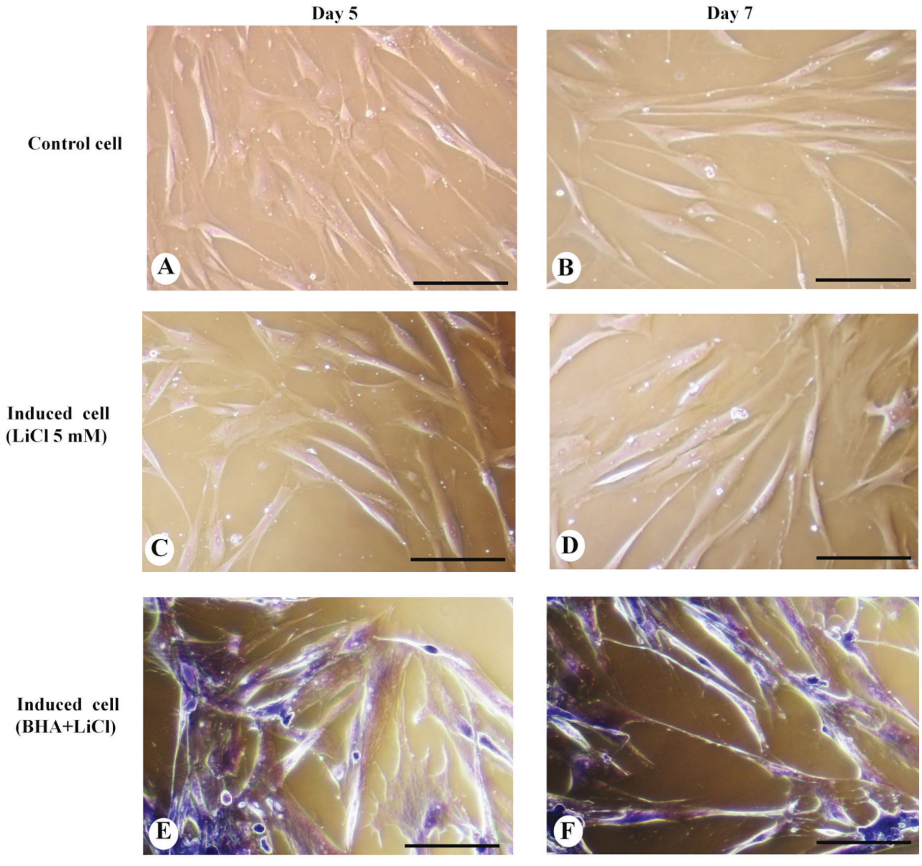

Figure 4 Demonstration of neural inductions by 2 different culture media. After treated WJMSCs with combination of BHA and LiCl (E, F), a small number of stem cells were changed to cells with a neural like cell phenotype when compared with control cell (A, B). However, LiCl-treated WJMSCs for 5 days (C) and 7 days (D), no change of stem cells morphology. Scale bar = 500 μm

The expression of neural marker genes was also demonstrated by using semi-quantitative RT-PCR technique. The expression level of *nestin* and β-*tubulin III* were increased from day 5th to day 7th when treated MSCs with LiCl alone and the combination of LiCl and BHA (Figure 5). Interestingly, LiCl-treated MSCs at day 7th of induction showed the highest expression of β-*tubulin III*. Although, LiCl alone and its combination with BHA could promote expression of *ngn1* at day 5th of induction, the expression of *ngn1* was decreased at day 7th.

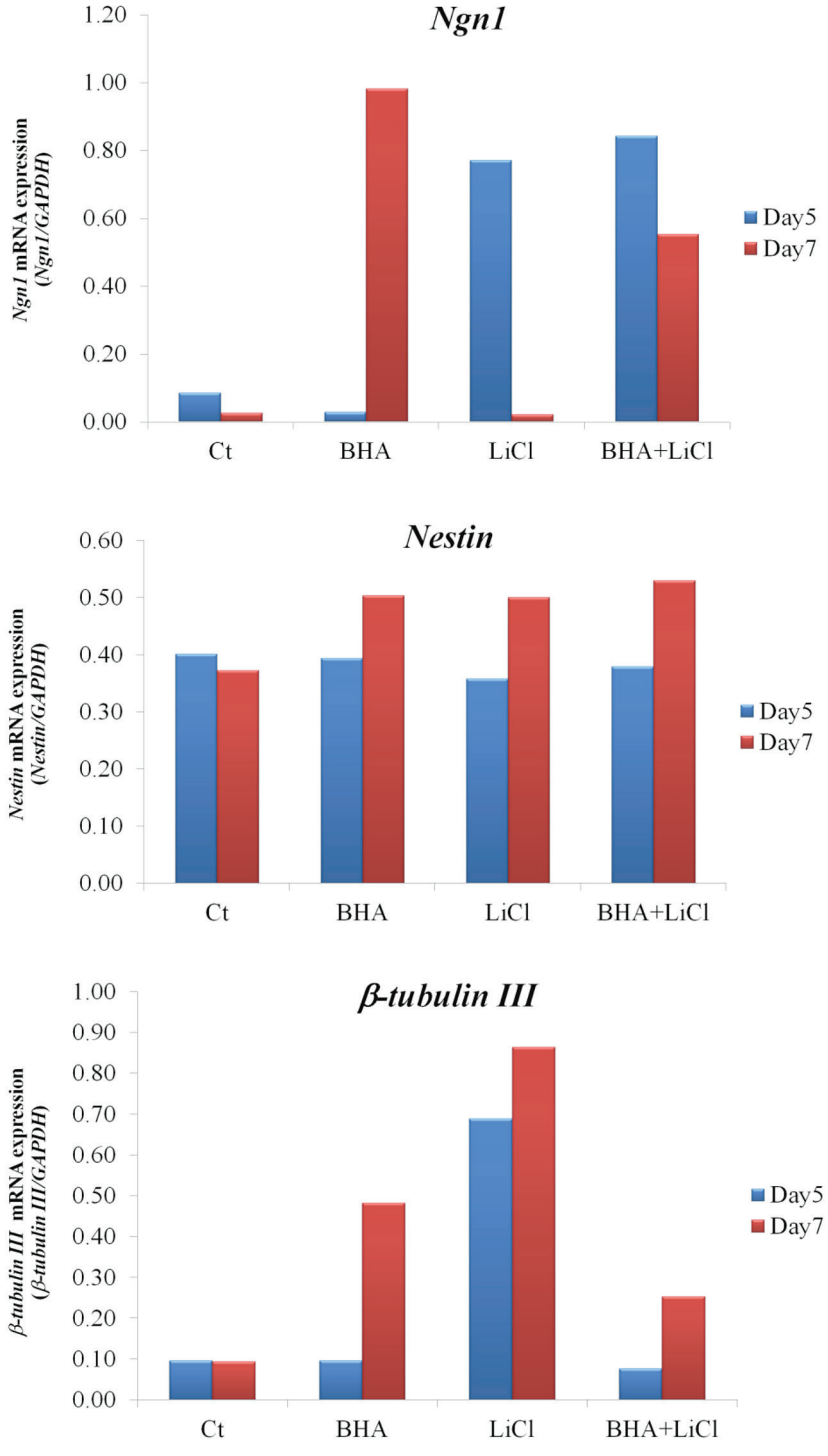

Figure 5 Neuronal gene expression of WJMSCs in neuronal medium containing BHA, LiCl and a combination of BHA and LiCl by semi-quantitative RT-PCR. The mRNA level relative to that of *GAPDH* was calculated. The results showed increased expression of all neural marker genes in BHA-treated cells. LiCl-treated cells or combined with BHA resulted in increased expression of *nestin* and β-*tubulin III* whereas *ngn1* expression was decreased.

Discussion and Conclusion

The properties of MSCs possess the potential to differentiate into many cell types. Thus, MSCs becomes attractive for therapeutic regenerative diseases. Many studies showed the similar neural differentiation potential from MSCs derived bone marrow, fat, spleen, thymus and umbilical cord blood.[12, 21 - 23] The study of Drela and colleague also demonstrated that WJMSCs express neuronal marker and glial marker higher than BM-MSCs.[21] Many studies suggested that MSCs are capable to differentiate into neural cells.[8, 9, 11] Woodbury and colleague demonstrated that rat MSCs and human bone marrow MSCs could be rapidly transformed into neural like cells after BHA treatment within 5 hours.[7] In our experiment, we observed the change of WJMSCs phenotype like neuron after treated MSCs with BHA for 5 days. MSCs from different sources were expressed different profiles of gene expression involved in cell function and gene in signaling pathways as previously reported.[21, 24] As the results, it is suggested that the different stem cell sources may response to stimuli in the different ways depending on the receptors and signaling proteins expression.

BHA induced transient neuron differentiation revealed that two cell populations were observed. The stem cells still maintain their phenotype although induction times are increased to 7 days. This finding suggesting that the culture parameters such as optimal time and dose of chemical medium might be optimized to improve the quality of neuron cell in further study. Neural gene expression was increases regarding to the induction time. Notably, the control sample, the expression of neural marker genes also found in untreated cells. This might possibly have occurred as the result of culture expansion of WJMSCs with serum enrichment medium prior to serum free culture containing various inducible growth factors that might act as neuron stimulators.[25]

Because of neural development involved in many signaling pathways including Wnt signaling,[15 - 17, 26 - 29] LiCl were used as a target chemical to promote Wnt signaling pathway in neural induction from WJMSCs. As the results, LiCl-treated MSCs at 7 days of induction showed the highest expression of β-*tubulin III*. This result suggested that Wnt signaling pathway may involve in β-*tubulin III* expression. However, LiCl-treated MSCs could not change their phenotype like neuron, but the other neural genes, *nestin* and β-*tubulin III* were increased at indicated time point. The controversial results possibly due to the fact that Wnt signaling pathway can crosstalk to other signaling pathways including TGF-β, MAPK, PPARs Alpha and Gamma.[19, 30-33] Therefore, understanding the signaling pathway underlies neural differentiation may promote both neural phenotypes along with neural gene expression. BHA and LiCl also affect in promoting *nestin* and β-*tubulin III* expression while they have the different effect in *ngn1* expression. Interestingly, the expression of this gene can be restored when treated MSCs with induction medium containing LiCl and BHA.

Our data indicated that neural induction from WJMSCs by BHA and LiCl could be possible because the morphology of MSCs and neural gene expression were changed after treatment with these chemicals. Further confirmation of neural differentiation is required the functional neuron in term of neurotransmitter release or electrophysiological properties. These results will lead to the effective culture process for neuronal induction from WJMSCs with high yield of neural like phenotype and neural gene expression for therapeutic regenerative disorders.

Limitations

This study was limited by the cytotoxic effects of BHA in combination with LiCl. Several reports examined that LiCl showed cytotoxic effect in a dose-and time-dependent manner.[19, 20, 34] Although, the previous study demonstrated that 200 μM BHA

and 5 mM LiCl each did not decrease the viability of stem cells.[7, 19, 20] The combination of 200 μM BHA and 5 mM LiCl might induce WJMSC death. This may affect the neuronal gene expression when culture stems cells for long term differentiation using medium containing BHA and LiCl. Therefore, BHA and LiCl should be further studied in future to evaluate the cytotoxic effect on WJMSCs.

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

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บทคัดย่อ

ประสิทธิภาพการเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์จาก Wharton's jelly เป็นเซลล์ประสาทด้วยสารลิเทียมคลอไรท์และสารบิวทิเลเทตไฮดรอกซีแอนิโซล

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บทนำ: สารกระตุ้นที่มีประสิทธิภาพในการเหนี่ยวนำเซลล์ต้นกำเนิดให้พัฒนาเป็นเซลล์ประสาทเป็นปัจจัยสำคัญต่อความสำเร็จในการรักษาโรคโดยใช้เซลล์ต้นกำเนิด โดยสารกระตุ้นที่ใช้กันอย่างแพร่หลายคือบิวทิเลเทตไฮดรอกซีแอนิโซล แต่การกระตุ้นโดยใช้สารนี้พบว่าหลังการเหนี่ยวนำยังมีเซลล์ต้นกำเนิดบางเซลล์ไม่สามารถพัฒนาเป็นเซลล์ประสาทได้ ส่วนงานวิจัยเกี่ยวกับกระบวนการพัฒนาของสมอง พบว่า Wnt signaling pathway มีบทบาทสำคัญในการควบคุมการพัฒนาของเซลล์ประสาท งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อประเมินประสิทธิภาพของสารลิเทียมคลอไรด์ซึ่งเป็นสารกระตุ้น Wnt signaling pathway และสารบิวทิเลเทตไฮดรอกซีแอนิโซลในการเหนี่ยวนำเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่แยกจาก Wharton's Jelly ให้พัฒนาเป็นเซลล์ประสาท

วิธีการศึกษา: เลี้ยงเซลล์ต้นกำเนิดในอาหาร ๓ สูตร คือ ๑) บิวทิเลเทตไฮดรอกซีแอนิโซล 200 μM ๒) ลิเทียมคลอไรด์ 5 mM ๓) บิวทิเลเทตไฮดรอกซีแอนิโซล 200 μM ผสมลิเทียมคลอไรด์ 5 mM เป็นเวลา ๗ วัน ย้อม hematoxylin เพื่อดูรูปร่างของเซลล์และตรวจการแสดงออกของยีนเซลล์ประสาท ๓ ยีนคือ *nestin* β-*tubulin III* และ *neurogenin* ด้วยเทคนิค semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

ผลการศึกษา: การเหนี่ยวนำเซลล์ต้นกำเนิดโดยใช้อาหารสูตรที่ ๑ และ ๓ ทำให้เซลล์เปลี่ยนแปลงรูปร่างไปคล้ายกับเซลล์ประสาท และยังพบว่าเซลล์ที่เลี้ยงในอาหารสูตรที่ ๑ มีการแสดงออกของยีนเซลล์ประสาททั้ง ๓ ยีนเพิ่มสูงขึ้น ส่วนเซลล์ที่เลี้ยงด้วยอาหารสูตรที่ ๒ และ ๓ พบการแสดงออกเฉพาะยีน *nestin* และ β-*tubulin III* เพิ่มสูงขึ้น

วิจารณ์ และสรุปผลการศึกษา: การศึกษาครั้งนี้แสดงให้เห็นถึงแนวทางในการใช้สารบิวทิเลเทตไฮดรอกซีแอนิโซลและลิเทียมคลอไรด์ในการเหนี่ยวนำเซลล์ต้นกำเนิดให้พัฒนาเป็นเซลล์ประสาทได้ อย่างไรก็ตามกลไกที่แท้จริงในการเหนี่ยวนำเซลล์ต้นกำเนิดให้พัฒนาเป็นเซลล์ประสาทด้วยสาร ๒ ชนิดนี้จำเป็นต้องศึกษาต่อไปในอนาคต

คำสำคัญ: เซลล์ต้นกำเนิดชนิดมีเซนไคม์จาก Wharton's Jelly, เซลล์ประสาท, ลิเทียมคลอไรด์, บิวทิเลเทตไฮดรอกซีแอนิโซล