INDUCTION OF HUMAN UMBILICAL CORD WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS INTO NEUROSPHERES AND TRANSPLANTATION FOR SPINAL CORD INJURY OF RAT MODEL

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การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่สายรกของมนุษย์เป็นเซลล์นิวโรสเฟียร์ และการปลูกถ่ายให้หนูแรทที่มีภาวะไขสันหลังฉีกขาด

นางสาวศิริลักษณ์ สำเร็จงาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ศิริลักษณ์ สำเร็จงาน : การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่สายรกของมนุษย์เป็นเซลล์นิวโรสเฟียร์ และการปลูกถ่ายให้หนูแรทที่มีภาวะไขสันหลังฉีกขาด (INDUCTION OF HUMAN UMBILICAL CORD WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS INTO NEUROSPHERES AND TRANSPLANTATION FOR SPINAL CORD INJURY OF RAT MODEL) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. รังสรรค์ พาลพ่าย, 83 หน้า.

คำสำคัญ: เซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่สายรกของมนุษย์/นิวโรสเฟียร์/การปลูกถ่าย/การฟื้นฟู/ภาวะไขสันหลังฉีกขาด

การบาดเจ็บไขสันหลังส่งผลให้เกิดการอักเสบและการตายของเซลล์ประสาทที่นำไปสู่การสูญเสียความสามารถในการเคลื่อนไหว เนื่องจากการรักษาภาวะการบาดเจ็บไขสันหลังที่ยังมีข้อจำกัด การรักษาด้วยเทคโนโลยีเซลล์ต้นกำเนิดบำบัดเป็นอีกทางออกหนึ่งในการรักษาโรคที่เกี่ยวกับระบบประสาทและการบาดเจ็บไขสันหลัง เซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่สายรกของมนุษย์ก็เป็นอีกทางเลือกหนึ่งสำหรับใช้ในเทคโนโลยีเซลล์ต้นกำเนิดบำบัด เนื่องจากง่ายที่จะขอรับบริจาคและไม่ต่อต้านระบบภูมิคุ้มกันหลังจากใช้ในการปลูกถ่าย โดยการทดลองครั้งนี้มีจุดประสงค์ที่จะนำเซลล์ต้นกำเนิดมีเซ็นไคม์ที่ได้มาเหนี่ยวนำให้เป็นเซลล์ประสาทต้นกำเนิด/เซลล์ประสาทโปรเจนิเตอร์ ในลักษณะเซลล์นิวโรสเฟียร์ด้วยน้ำยาเหนี่ยวนำที่มีสารชีวโมเลกุลขนาดเล็กคือ P7C3-A20 และ Isx9 แล้วปลูกถ่ายในหนูแรทที่ถูกทำให้เกิดการบาดเจ็บของไขสันหลัง เซลล์นิวโรสเฟียร์ที่ได้ถูกนำไปตรวจสอบโปรตีนที่จำเพาะต่อเซลล์นิวโรสเฟียร์ด้วยการย้อมสีฟลูออเรสเซนต์ การแยกโปรตีนในเจลและตรวจสอบการแสดงออกของยีน ผลการตรวจสอบการแสดงออกของโปรตีนและยีนพบว่าเซลล์นิวโรสเฟียร์ของกลุ่มที่เพิ่มสารชีวโมเลกุลขนาดเล็ก Isx9 ที่ความเข้มข้น 10 μM ลงในน้ำยาเหนี่ยวนำเป็นระยะเวลา 7 วันได้แสดงตัวบ่งชี้ที่จำเพาะและการแสดงออกของยีนที่จำเพาะคือ nestin และ β-tubulin 3 สำหรับเซลล์ประสาทต้นกำเนิด/เซลล์ประสาทโปรเจนิเตอร์ นอกจากนี้ แสดงตัวบ่งชี้ที่จำเพาะและการแสดงออกของยีนที่จำเพาะ β-catenin และ NEUROD1 จากการเหนี่ยวนำไปเป็นเซลล์ประสาทต้นกำเนิดผ่านการสื่อสัญญาณภายในเซลล์ที่เรียกว่า Wnt3A signaling pathway จากนั้นเซลล์นิวโรสเฟียร์กลุ่ม 10 μM Isx9 ที่ถูกเหนี่ยวนำเป็นระยะเวลา 7 วัน ถูกนำไปนำไปใช้ในการปลูกถ่ายในหนูแรทที่ถูกทำให้เกิดการบาดเจ็บของไขสันหลังแล้วเป็นระยะเวลา 9 วัน หลังจากปลูกถ่ายเซลล์ไปแล้ว 8 สัปดาห์หนูที่ถูกปลูกถ่ายด้วยเซลล์นิวโรสเฟียร์สามารถเคลื่อนที่ได้ใกล้เคียงกับปกติมากที่สุดจากผลทดสอบการทำงานของเนื้อเยื่อไขสันหลัง ส่วนผลทางเนื้อเยื่อวิทยาพบว่ามีการอยู่รอดของเซลล์ที่ใช้ปลูกถ่ายเข้าไปในเนื้อเยื่อไขสันหลังหนูแรททั้งกลุ่มเซลล์ต้นกำเนิดมีเซ็นไคม์และกลุ่มเซลล์นิวโรสเฟียร์พร้อมทั้งแสดงออกถึงโปรตีนของเซลล์

ประสาท (β-tubulin 3) และมีการทำงานของสารสื่อประสาท (ChAT) นอกจากนี้กลุ่มที่ปลูกถ่ายด้วยเซลล์นิวโรสเฟียร์ยังพบปริมาตรพื้นที่ของเนื้อเยื่อที่บาดเจ็บบริเวณเนื้อเยื่อไขสันหลังที่น้อยที่สุดที่แสดงถึงการมีกระบวนการฟื้นฟูของเนื้อเยื่อไขสันหลังเกิดขึ้น

การทดลองครั้งนี้สรุปได้ว่า เซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่สายรกของมนุษย์สามารถถูกเหนี่ยวนำไปเป็นเซลล์นิวโรสเฟียร์ในน้ำยาที่ประกอบด้วย 10 μM Isx9 ผ่าน Wnt3A signaling pathway เป็นระยะเวลา 7 วัน และหลังจากปลูกถ่ายเซลล์นิวโรสเฟียร์เข้าสู่หนูแรทที่ทำให้เกิดภาวะไขสันหลังฉีกขาดเป็นระยะเวลา 8 สัปดาห์ พบว่าเซลล์นิวโรสเฟียร์ที่ได้มีความสามารถที่จะเปลี่ยนแปลงไปเป็นเซลล์ประสาทพร้อมกับการทำงานของสารสื่อประสาทได้ซึ่งสามารถฟื้นฟูและรักษาอาการบาดเจ็บไขสันหลังในหนูแรทได้ดีกว่าอย่างชัดเจนเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้ปลูกถ่าย

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2565

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SIRILAK SOMREDNGAN : INDUCTION OF HUMAN UMBILICAL CORD WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS INTO NEUROSPHERES AND TRANSPLANTATION FOR SPINAL CORD INJURY OF RAT MODEL. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 83 PP.

Keyword: HUMAN UMBILICAL CORD WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS/NEUROSPHERE/TRANSPLANTATION/RECOVERY/SPINAL CORD INJURY

Spinal cord injury (SCI) causes inflammation and neuron degeneration, leading to loss of functional movement. Since the availability of SCI treatments are still limited, stem cell therapy is an alternative clinical treatment for SCI and neurodegeneration disorders. Human umbilical cord Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) are excellent options for cell therapy, since they are simple to deliver and have not shown any negative impacts on allogeneic transplantation. This study aims to induce hWJ-MSCs into neural stem/progenitor cells in sphere formation (neurosphere) by using neurogenesis-enhancing small molecules (P7C3 and Isx9) and transplant to recover SCI in rat model. Neurospheres induction were characterized by immunocytochemistry (ICC), western blot, and gene expression analysis before transplantation. The result of protein and gene expression showed that neurospheres, induced by 10 μM Isx9 for 7 days, produced neural stem/progenitor cell markers such as nestin and β-tubulin 3. Additionally, neural stem cells differentiation through Wnt3A signaling pathway regulation marker (β-catenin and NEUROD1) were also expressed. The neurospheres from the 7-day Isx9 group were selected to be transplanted into 9-day SCI rats. Eight weeks after transplantation, rats transplanted with the neurospheres could move normally, as shown by behavioral tests. Histological results showed that MSCs and neurospheres were detected in the injured spinal cord tissue and can produce neurotransmitter activity. The neurosphere transplanted rats showed the lowest cavity size of the SCI tissue resulted from injury recovery mechanism in the spinal cord.

In conclusion, hWJ-MSCs could differentiate into neurospheres using 10 μM Isx9 media through Wnt3A signaling pathway. Eight weeks after transplantation, neurospheres

could be detected and differentiated into neural lineage with neurotransmission function. The SCI rats with neurospheres transplant had better recovery of the SCI tissue and movement than the SCI rats without transplantation of neurospheres.

School of Biotechnology
Academic Year 2022

Student's Signature
Advisor's Signature
Co-advisor's Signature

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LIST OF ABBREVIATIONS

α-MEM	=	alpha modification of Eagle's medium
ACh	=	acetylcholine
ANOVA	=	one-way analysis of variance
BDNF	=	brain-derived neurotrophic factor
bFGF	=	basic fibroblast growth factor
BSA	=	bovine serum albumin
cDNA	=	complementary DNA
DAB	=	3,3'-diaminobenzidine tetrachloride
DAPI	=	6-diamino-2-phenylindole
DMSO	=	dimethyl sulfoxide
ECL	=	enhanced chemiluminescence
EDTA	=	ethylene-di-amino-tetra-acetic-acid
EGF	=	epidermal growth factor
ESC-NSs	=	embryonic stem cell-derived neurospheres
FBS	=	fetal bovine serum
GAG	=	glycosaminoglycans
H&E	=	hematoxylin and eosin
I.O.D.	=	integrated optical density
hDPSCs	=	human dental pulp stem cells
HIER	=	heat-Induced epitope retrieval
hiPSC-NSs	=	human induced pluripotent stem cell-derived neurospheres
HRP	=	horseradish peroxidase
HSD	=	Tukey-Kramer Honest significant difference
hUC-MSCs	=	human umbilical cord mesenchymal stem cells
hWJ-MSCs	=	human Wharton's jelly mesenchymal stem cells
IBMX	=	isobutyl methylxanthine
ICC	=	immunocytochemical staining
Isx9	=	isoxazole 9

LIST OF ABBREVIATIONS (Continued)

ITS-X	=	insulin-transferrin-selenium-ethanolamine
LFB	=	luxol fast blue solution
MSCs	=	mesenchymal stem cells;
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NM	=	neurosphere culture medium
NSCs	=	neural stem cells
NSs	=	neurospheres
PBS(-)	=	phosphate-buffered saline minus
PFA	=	paraformaldehyde
PVDF	=	Polyvinyliden fluoride
RT	=	room temperature
SCI	=	spinal cord injury
SD	=	Sprague Dawley
SDS	=	sodium dodecyl sulphate
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T9	=	thoracic level 9
WHO	=	world health organization

CHAPTER I

INTRODUCTION

1.1 Background and significance

Every year, between 250,000 and 500,000 people around the world (World Health Organization; WHO) suffer from spinal cord injuries (SCI). Generally, spinal cords are injured by mechanical factors, including falls, motor vehicle accidents, sports injuries, work-related injuries, and community violence. After that, pathological mechanisms start and are followed by inflammation and cell degeneration in the injured region (Leung, 2012). SCI pathology can be divided into two phases. The primary phase begins with a direct traumatic effect on the neural cells and vascular structures of the spinal cord. The secondary phase is composed of inflammatory activation leading to cellular apoptosis, necrosis, glial scar formation and prolonged Wallerian degeneration. This degeneration process happens whenever a nerve fiber is cut or crushed at the axon distal to the injury part (Adeeb et al., 2014). Normally, steroids are used with SCI patients to limit existing cell death. To date, stem cell therapy plays an essential role in the clinical treatment of neurodegeneration disorders because stem cells have high proliferative potential, self-renewal, and the ability to differentiate into various types of cells (Ruppert et al., 2019).

Mesenchymal Stem Cells (MSCs) are multipotent stem cells that have the potential to multiply on their own, together with the ability to differentiate into many different types of cells, for example, bone cells, cartilage cells, liver cells, pancreatic cells, fat cells, neuronal and glial cells (Hess and Borlongan, 2008; Song et al., 2018). MSCs also have immunosuppressive properties, which is an important property for the allogeneic cell therapy process (Pelizzo et al., 2015; Toyoshima et al., 2015; Xue et al., 2015). MSCs are found in many tissues, for example, the umbilical cord, bone marrow, placenta, and adipose tissue (Venkatesh and Sen, 2017). However, those tissue samples might provide a small number of cells and low proliferation potential depending on age. In addition, a high chance of getting

infected with viruses can be found (Goodwin et al., 2001; Kakinuma et al., 2007; Rao and Mattson, 2001). On the other hand, MSCs derived from human Wharton's jelly (hWJ-MSCs) are easy to obtain and usually discarded. hWJ-MSCs also have a high proliferation capability. In case of transplantation, hWJ-MSCs do not change into carcinogenic or teratogenic cells and are non-tumorigenic (Fong et al., 2007; Ranjbaran et al., 2018). Besides, hWJ-MSCs demonstrated the characteristics of both embryonic stem cells and adult stem cell properties. Thus, they could be a suitable source for therapeutic use of stem cells (Carlin et al., 2006).

MSCs can be transdifferentiated into neurospheres (NSs). NSs are spheres formed of neural stem/progenitor cell aggregates with the ability to differentiate into neuronal lineage (Bonnamain et al., 2011; Muniswami et al., 2017). In previous literature, NSs from fetal spinal cord cultures were transplanted and treated for SCI in rats (Ogawa et al., 2002). Furthermore, SCI rats transplanted by the induction of neural stem/progenitor cells showed improved functional development following SCI recovery (Parr et al., 2008).

A lot of small molecules were used to induce human skin fibroblasts into neurons. P7C3-20 and Isx9 demonstrated high efficiency for neuron induction, as previously reported (Yang et al., 2019). P7C3-A20 has been found to activate phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/glycogen synthase kinase (GSK) and to be involved in β-catenin signaling. (Kawase-Koga et al., 2020). PI3K/AKT pathway also induced cell proliferation, differentiation, and apoptosis, while GSK, by serine/threonine-protein kinase, has been shown to induce apoptosis prevention in brain injury models (Hosseini et al., 2018). Second small molecule, isoxazole 9 (Isx9; N-cyclopropyl-5-(thiophene-2-yl) isoxazole-3-carboxamide) strongly induced adult neural stem cells to neuronal linage *in vitro* (Shohayeb et al., 2018). Furthermore, Isx9 stimulated the myocyte-enhancer factor 2, a family of transcription factors (Vanderhaeghen, 2009), which impacts to embryo and early post-natal period as pro-neuronal and pro-survival factors (Hagihara et al., 2019). *In vivo*, Isx9 also induced hippocampal neurogenesis, development, and memory by regulating the cell-intrinsic molecular pathway in adult neurogenesis (Peng et al., 2019).

In this study, hWJ-MSCs were differentiated into NSs using small molecules to regulate and enhance the neurogenesis. This would be an appropriate protocol for the therapy of neurodegenerative disorders.

1.2 Research objective

1.2.1 To isolate and culture hWJ-MSCs.

1.2.2 To differentiate hWJ-MSCs into NSs via neurogenesis enhancing small molecules *in vitro*.

1.2.3 To transplant NSs into spinal cord injury animal model to test the ability and safety of the treatment.

1.3 References

Adeeb, N., Hose, N., Tubbs, R. S., & Mortazavi, M. (2014). Stem cell therapy for spinal cord injury: cellular options. **Austin Journal of Cerebrovascular Disease & Stroke**, *1*, 5.

Bonnamain, V., Neveu, I., & Naveilhan, P. (2011). *In vitro* analyses of the immunosuppressive properties of neural stem/progenitor cells using anti-CD3/CD28-activated T cells. **Methods in Molecular Biology**, *677*, 233-243. doi:10.1007/978-1-60761-869-0_17.

Carlin, R., Davis, D., Weiss, M., Schultz, B., & Troyer, D. (2006). Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. **Reproductive Biology and Endocrinology**, *4*(1), 8. doi:10.1186/1477-7827-4-8.

Fong, C. Y., Richards, M., Manasi, N., Biswas, A., & Bongso, A. (2007). Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. **Reproductive BioMedicine Online**, *15*(6), 708-718. doi:10.1016/s1472-6483(10)60539-1.

Goodwin, H. S., Bicknese, A. R., Chien, S. N., Bogucki, B. D., Quinn, C. O., & Wall, D. A. (2001). Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. **Biology of Blood**

and Marrow Transplantation, *7*(11), 581-588. doi:10.1053/bbmt. 2001.v7. pm11760145.

Hagihara, H., Murano, T., Ohira, K., Miwa, M., Nakamura, K., & Miyakawa, T. (2019). Expression of progenitor cell/immature neuron markers does not present definitive evidence for adult neurogenesis. **Molecular Brain**, *12*(1), 108. doi:10.1186/s13041-019-0522-8.

Hess, D. C., & Borlongan, C. V. (2008). Stem cells and neurological diseases. **Cell Proliferation**, *41 Suppl 1*(Suppl 1), 94-114. doi:10.1111/j.1365-2184.2008. 00486.x.

Hosseini, S. M., Sani, M., Haider, K. H., Dorvash, M., Ziaee, S. M., Karimi, A., & Namavar, M. R. (2018). Concomitant use of mesenchymal stem cells and neural stem cells for treatment of spinal cord injury: A combo cell therapy approach. **Neuroscience Letters**, *668*, 138-146. doi:10.1016/j.neulet.2018.01.008.

Kakinuma, S., Asahina, K., Okamura, K., Teramoto, K., Tateno, C., Yoshizato, K., Tanaka, Y., Yasumizu, T., Sakamoto, N., Watanabe, M., & Teraoka, H. (2007). Human cord blood cells transplanted into chronically damaged liver exhibit similar characteristics to functional hepatocytes. **Transplantation Proceedings**, *39*(1), 240-243. doi:10.1016/j.transproceed.2006.10.211.

Kawase-Koga, Y., Fujii, Y., Yamakawa, D., Sato, M., & Chikazu, D. (2020). Identification of neurospheres generated from human dental pulp stem cells in xeno-/serum-free conditions. **Regenerative Therapy**, *14*, 128-135. doi:10.1016/ j.reth.2019.11.006.

Leung, L. (2012). Cellular therapies for treating pain associated with spinal cord injury. **Journal of Translational Medicine**, *10*(1), 37. doi:10.1186/1479-5876-10-37.

Muniswami, D. M., Kanakasabapathy, I., & Tharion, G. (2017). Globose basal cells for spinal cord regeneration. **Neural Regeneration Research**, *12*(11), 1895-1904. doi:10.4103/1673-5374.219052.

Ogawa, Y., Sawamoto, K., Miyata, T., Miyao, S., Watanabe, M., Nakamura, M., Bregman, B. S., Koike, M., Uchiyama, Y., Toyama, Y., & Okano, H. (2002). Transplantation of *in vitro*-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. **Journal of Neuroscience Research**, *69*(6), 925-933. doi:10.1002/jnr.10341.

Parr, A. M., Kulbatski, I., Zahir, T., Wang, X., Yue, C., Keating, A., & Tator, C. H. (2008). Transplanted adult spinal cord-derived neural stem/progenitor cells promote early functional recovery after rat spinal cord injury. **Neuroscience**, *155*(3), 760-770. doi:10.1016/j.neuroscience.2008.05.042.

Pelizzo, G., Avanzini, M. A., Icaro Cornaglia, A., Osti, M., Romano, P., Avolio, L., Maccario, R., Dominici, M., De Silvestri, A., Andreatta, E., Costanzo, F., Mantelli, M., Ingo, D., Piccinno, S., & Calcaterra, V. (2015). Mesenchymal stromal cells for cutaneous wound healing in a rabbit model: pre-clinical study applicable in the pediatric surgical setting. **Journal of Translational Medicine**, *13*, 219. doi:10.1186/s12967-015-0580-3.

Peng, C., Li, Y., Lu, L., Zhu, J., Li, H., & Hu, J. (2019). Efficient one-step induction of human umbilical cord-derived mesenchymal stem cells (UC-MSCs) produces msc-derived neurospheres (MSC-NS) with unique transcriptional profile and enhanced neurogenic and angiogenic secretomes. **Stem Cells International**, *2019*, 9208173. doi:10.1155/2019/9208173.

Ranjbaran, H., Abediankenari, S., Mohammadi, M., Jafari, N., Khalilian, A., Rahmani, Z., Momeninezhad Amiri, M., & Ebrahimi, P. (2018). Wharton's jelly derived-mesenchymal stem cells: isolation and characterization. **Acta Medica Iranica**, *56*(1), 28-33.

Rao, M. S., & Mattson, M. P. (2001). Stem cells and aging: expanding the possibilities. **Mechanisms of Ageing and Development**, *122*(7), 713-734. doi:10.1016/s0047-6374(01)00224-x.

Ruppert, K. A., Olson, S. D., & Cox, C. S. (2019). Chapter 13 - spinal cord injury. In X.-D. Chen (Ed.), **A Roadmap to Non-Hematopoietic Stem Cell-based Therapeutics** (pp. 321-343): Academic Press.

Shohayeb, B., Diab, M., Ahmed, M., & Ng, D. C. H. (2018). Factors that influence adult neurogenesis as potential therapy. **Translational Neurodegeneration**, *7*(1), 4. doi:10.1186/s40035-018-0109-9.

Song, C. G., Zhang, Y. Z., Wu, H. N., Cao, X. L., Guo, C. J., Li, Y. Q., Zheng, M. H., & Han, H. (2018). Stem cells: a promising candidate to treat neurological disorders. **Neural Regeneration Research**, *13*(7), 1294-1304. doi:10.4103/1673-5374.235085.

Toyoshima, A., Yasuhara, T., Kameda, M., Morimoto, J., Takeuchi, H., Wang, F., Sasaki, T., Sasada, S., Shinko, A., Wakamori, T., Okazaki, M., Kondo, A., Agari, T., Borlongan, C. V., & Date, I. (2015). Intra-arterial transplantation of allogeneic mesenchymal stem cells mounts neuroprotective effects in a transient ischemic stroke model in rats: analyses of therapeutic time window and its mechanisms. **Plos One**, *10*(6), e0127302. doi:10.1371/journal.pone.0127302.

Vanderhaeghen, P. (2009). Wnts blow on NeuroD1 to promote adult neuron production and diversity. **Nature Neuroscience**, *12*(9), 1079-1081. doi:10.1038/nn0909-1079.

Venkatesh, K., & Sen, D. (2017). Mesenchymal stem cells as a source of dopaminergic neurons: a potential cell based therapy for parkinson's disease. **Current Stem Cell Research and Therapy**, *12*(4), 326-347. doi:10.2174/1574888x12666161114122059.

Xue, X., Liu, Y., Zhang, J., Liu, T., Yang, Z., & Wang, H. (2015). Bcl-xL genetic modification enhanced the therapeutic efficacy of mesenchymal stem cell transplantation in the treatment of heart infarction. **Stem Cells International**, *2015*, 176409. doi:10.1155/2015/176409.

Yang, Y., Chen, R., Wu, X., Zhao, Y., Fan, Y., Xiao, Z., Han, J., Sun, L., Wang, X., & Dai, J. (2019). Rapid and efficient conversion of human fibroblasts into functional neurons by small molecules. **Stem Cell Reports**, *13*(5), 862-876. doi:10.1016/j.stemcr.2019.09.007.

CHAPTER II
LITERATURE REVIEW

2.1 Wharton's jelly mesenchymal stem cells

Stem cells have a critical role in medical cell therapy research because of the fact that stem cells are immature cells that have self-renewal ability. Stem cells could be regenerated into various cell types. Stem cells have three main characteristics, (1) unspecialization: the ability to remain undifferentiated in the absence of specialized responsibilities, (2) differentiation: the capacity to give rise to numerous cell categories and (3) self-renewal: the ability to proliferation with same ability (Condic, 2014; Gardner, 2002; Katari and Dwaipayan, 2017; Mitalipov & Wolf, 2009). Adult stem cells are multipotent endogenous stem cells that are crucial to the development of stem cell therapy and tissue engineering. Because they are frequently used to repair and maintain the homeostasis of endogenous stem cells (Katari and Dwaipayan, 2017).

MSCs are multipotent stem cells that can be isolated from, for example, umbilical cord blood, Wharton's jelly, bone marrow, placenta, adipose tissue (Katari and Dwaipayan, 2017). Because of ethical and safety concerns regarding the use of bone marrow or other resources, MSCs from Wharton's jelly of umbilical cord are the easiest to approach and obtain because they are typically discarded as human unused. Furthermore, hWJ-MSCs have a high proliferation valency, and when transplanted, hWJ-MSCs do not transform into carcinogenic or teratogenic cells, and are non-tumorigenic (Fong et al., 2007; Ranjbaran et al., 2018). Furthermore, hWJ-MSCs are not embryonic stem cells (ESCs), they are adult stem cells (ASCs). Therefore, their properties can be a suitable source for the therapeutic use of stem cells (Carlin et al., 2006).

Differentiation, paracrine influence, immune-modulation, and anti-inflammatory actions are the four therapeutic uses of MSCs. According to a previous study, trophic factors secreted by MSCs help to regenerate and repair injured cells. These outcomes are one of the advantages of MSCs for transplantation models. MSCs in

damaged tissues have also been shown to repair them through differentiation and paracrine signaling (Hocking and Gibran, 2010; Katari and Dwaipayan, 2017). MSC transplantation has been reported to be used as therapy for various diseases. For example, myocardial infracted animal models showed considerable improvement in the heart's left ventricular ejection percentage (Berry et al., 2006). In cutaneous wounds, MSCs have been examined for their ability to differentiate into epidermal keratinocytes, endothelial cells, and pericytes (Wu et al., 2007) and improved myofibroblast differentiation in wound healing (Desai et al., 2014). Moreover, hematopoietic stem cells (HSCs) were helped to survive following transplantation by MSCs. Additionally, immune suppression establishes high graft survival for stem cell treatment and tissue regeneration investigations by inhibition of T and B cell proliferation and NK cell activity (Katari and Dwaipayan, 2017; Miguel et al., 2012). Also, MSCs improved the process of nerve cell differentiation and axonal remyelination (Cruz-Martinez et al., 2016).

2.2 Neurosphere

Neural stem cells (NSCs) are derived from a variety of tissues during the embryonic phases of the nervous system, including the subventricular zone of the forebrain, bone marrow, skin, and retina to generate new neurons (Kawase-Koga et al., 2020). NSCs only have a limited capacity to regenerate neuroglia in the adult mammalian brain. Therefore, stem cells are a suitable replacement therapy alternative for illnesses or disorders (George et al., 2019). NSCs are one of the most important treatments for central and peripheral nervous system lesions. For example, SCI, stroke, and Alzheimer's disease (Jung et al., 2016; Mouhieddine et al., 2014). Alternatively, neural progenitor cell (NPC) differentiation might be achieved by supplementing cell culture growth with small molecules that imitate the microenvironment of the brain during organogenesis (George et al., 2019).

In vitro, MSCs are stimulated to differentiate into NSs in a serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (George et al., 2019). Besides, NSs could be differentiated into neurons, astrocytes, and oligodendrocytes with cell migration out of adherent conditions for long-term

culture (Bonnamain et al., 2012; Bonnamain et al., 2011). NSs are a combination of NSCs and NPCs with inappropriate environmental conditions called Neural Stem/Progenitor Cells (NSPCs) (Kawase-Koga et al., 2020). NSPCs are multipotent stem cells with the potential to self-renew and develop into lineage-specific neural precursor cells, which may then give rise to neurons, astrocytes, and oligodendrocytes (Salewski et al., 2013). Several researchers are interested in NSs induction for spinal cord injury transplantation, such as from ESCs (Hosseini et al., 2018; Silva, et al., 2017), induced pluripotent stem cells (iPSCs) (Nori et al., 2018; Nori et al., 2015; Nori et al., 2011) or ASCs (Biernaskie et al., 2007; Cizkova et al., 2018; Hosseini et al., 2018; Muniswami et al., 2017; Nori et al., 2015). ASCs are multipotent stem cells and can produce neural cells in microenvironment culture conditions. There are numerous studies about NSs induced by ASCs. Human dental pulp stem cells (hDPSCs) were induced to be NSs in a proliferative medium lacking of Xeno/serum (Kawase-Koga et al., 2020). The results showed the NSs had limited self-renewal ability but the expression of β-tubulin 3 and microtubule-associated protein (MAP2) was more significantly expressed in NSs derived from hDPSCs. Within 12 hours, NSs were also produced from human umbilical cord-derived mesenchymal stem cells (UC-MSCs) in the conditions of DMEM/F12, EGF, bFGF, N2, and B27 (Peng et al., 2019). These NSs were capable of self-renewal and differentiation into neurons and glial cells in an inducing medium. In addition, NSs produced from UC-MSCs presented a comparable transcriptional profile to UC-MSCs and NSCs. Thus, NSs produced from UC-MSCs may establish a unique entity of stem cells. Additionally, HLA-DR expression was not found in these NSs, which can be securely used in transplantation therapy.

In the NSs culture medium, B27 and N2 are cocktail chemicals frequently utilized for NSs production by the peripheral nervous system (PNS) and the central nervous system (CNS). Insulin, human transferrin (iron-saturated), sodium selenite, putrescine, and progesterone are included in the N2 supplement. The B27 supplement includes lengthy lists of vitamins, hormones, proteins, and others. N2 cannot stimulate NSs production on its own, but B27 supplement components may play a significant role in this reaction. According to a recent study, UC-MSCs may be encouraged to produce neurons under conventional culture conditions (DMEM/F12, EGF, bFGF, N2, and B27) after 12 hours. They concluded the simple induction

method for NSs (1) The induction period is brief, lasting only a few hours. (2) The induction is extraordinarily effective. It is possible to induce 80-92 percent of MSCs to differentiate into NSs. (3) The induction method is very simple and clearly explained. Only the standard NSs culture medium is used. For the generation of NSs, it is not essential to overexpress or downregulate specific genes via retro-lentiviral vector intervention. Consequently, NSs were capable of self-renewal and could be stimulated to differentiate into neurons. In the part of gene expression, the results showed the high expression of *SOX2* and *nestin* (multiple neurogenic genes) after being induced. On the one hand, strong expression of stemness genes such as *OLIG2*, *OCT4*, and *BMI1* was observed at day 1, 3, and 5 and 7 post-induction. As shown by an RNA sequencing study, the transcriptional pattern of NSs differs from both MSCs and human neural stem cells. In addition, the NSs have a relatively lower level of apoptosis (Peng et al., 2019).

The Infinite Horizon Impactor was used to generate spinal cord injury in rats as part of a study on transplantation treatment (Sontag et al., 2014). Nine days after injury, NSs derived from human CNS stem cells were transplanted. Although overall cell survival was reduced in the injured area, the number of mature oligodendrocytes and the astrocyte-to-oligodendrocyte ratio increased. In addition, rat olfactory epithelium cells were induced to form globose basal cells (GBCs) in the formation of NSs and transplanted into the injured rat spinal cord (contusion model) (Muniswami et al., 2017). The results showed the hindlimb motor recovery in rats at the end of the 8th week after transplantation.

2.3 Enhancing NSs development by small molecules

2.3.1 Small molecule P7C9-A20

It has been reported that the small molecule chemical P7C3 displays neuroprotective effects in the hippocampus of mice and in many preclinical models, including ischemic stroke, traumatic brain injury, peripheral nerve damage, and neurodegenerative disease models (Loris et al., 2017). P7C3 inhibits cell death by targeting the nicotinamide phosphoribosyl transferase-mediated nicotinamide adenine dinucleotide salvage pathway (Wang et al., 2014). P7C3 modifies the

structure of the glucagon-like peptide-1 receptor. The P7C3 analog P7C3-A20 was shown to promote phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/glycogen synthase kinase (GSK) 3b-associated b-catenin signaling (Wang et al., 2018). The PI3K/AKT pathway promotes cell proliferation, differentiation, senescence, and apoptosis (Li et al., 2018), while GSK3b, a functional protein with different activities, has been shown to stop apoptosis in brain damage models.

2.3.2 Small molecule Isx9

Isoxazole 9 [Isx-9; N-cyclopropyl-5-(thiophene-2-yl) isoxazole-3-carboxamide] was discovered to be highly effective in stimulating neuronal differentiation *in vitro* (Schneider et al., 2008). In addition, Isx-9 interacts with myocyte-enhancer factor 2 (Mef2), a family of transcription factors (Potthoff and Olson, 2007) identified as pro-neuronal and pro-survival factors in the embryo and early postnatal period (Li et al., 2008). Isx-9 also improves hippocampus neurogenesis and memory *in vivo.* This shows that a cell-intrinsic molecular mechanism controls adult neurogenesis (Petrik et al., 2012).

2.4 Spinal cord injury transplantation

2.4.1 Pathology and stages of spinal cord injury

The pathophysiology of SCI is separated into two phases: the main stage and the sequel stage. Several events have been shown, such as inflammation and reactive astrogliosis, as well as neuroprotective and restorative effects. The primary stage is caused by initial mechanical damage followed by cellular and molecular disruptions and further spinal cord material degradation. However, complete spinal cord transections seldom involve mechanical injury. Most injuries are caused by disk displacement in the spine, laceration or compression from fracture-dislocations or burst fractures, or both. Moreover, blood vessel, axonal, and cellular membrane disruptions lead to the secondary stage by reducing blood flow to the tissue and cells of the spinal cord, which results in tissue necrosis due to a lack of nutrients and oxygen (Rowland et al., 2008; Ruff and Fehlings, 2010; Tator and Fehlings, 1991). Therefore, the pathological stage of SCI is characterized by vascular malfunction and bleeding, edema, ischemia, excitotoxicity, electrolyte

changes, free radical production, inflammation, axonal and neuronal degradation, demyelination, cyst development, and infarction (Tator and Fehlings, 1991). In the cellular damage level of SCI, there are five major stages: the immediate, acute, subacute, intermediate, and chronic stages (Salewski et al., 2013) (Fig. 2.1)

1) Immediate stage

Axon destruction, neuronal and glial death, and spinal shock were discovered in the first two hours following SCI. All of these immediate damages resulted in loss of function in the lower body. Since cellular necrosis, vascular disruption, and ischemia, there has been hemorrhage in the grey matter of the spinal cord. Vascular disruption causes white matter bleeding after the combination of edema and hemorrhage on multiple spinal cord segments. The damage occurs in both the rostral and caudal and leads to ischemic injury (Kakulas, 2004). Numerous pathophysiological processes have already started at this stage. Gross histopathological abnormalities, such as microglial cell activation, a rapid rise in pro-inflammatory cytokines like TNF and IL, and excitotoxic rates of extracellular glutamate, may not be detected (Pineau and Lacroix, 2007; Rowland et al., 2008; Tator and Koyanagi, 1997).

2) Acute stage

The acute phase of SCI is classified into early acute (2–48 hours) and subacute stages (2 days-2 weeks). In the early acute stages, the loss of homeostasis in the cell led to vascular damage, bleeding, and lack of blood flow (Tator and Fehlings, 1991; Tator and Koyanagi, 1997). Immediate loss of ionic homeostasis and excitotoxicity, as well as the homeostasis of ionic (calcium, sodium, and potassium) channels, FAS ligand receptors, p75 neurotrophin receptors, reactive oxygen species (ROS), the brain blood barrier (BBB), and inflammation, all play significant roles in necrosis cell and apoptotic cell death. For example, extracellular levels of glutamate cause cell damage and energy-dependent transporter failure, specifically in the Na^+ K^+ adenosine triphosphatase membrane transporter that controls extracellular concentration (Lipton and Rosenberg, 1994).

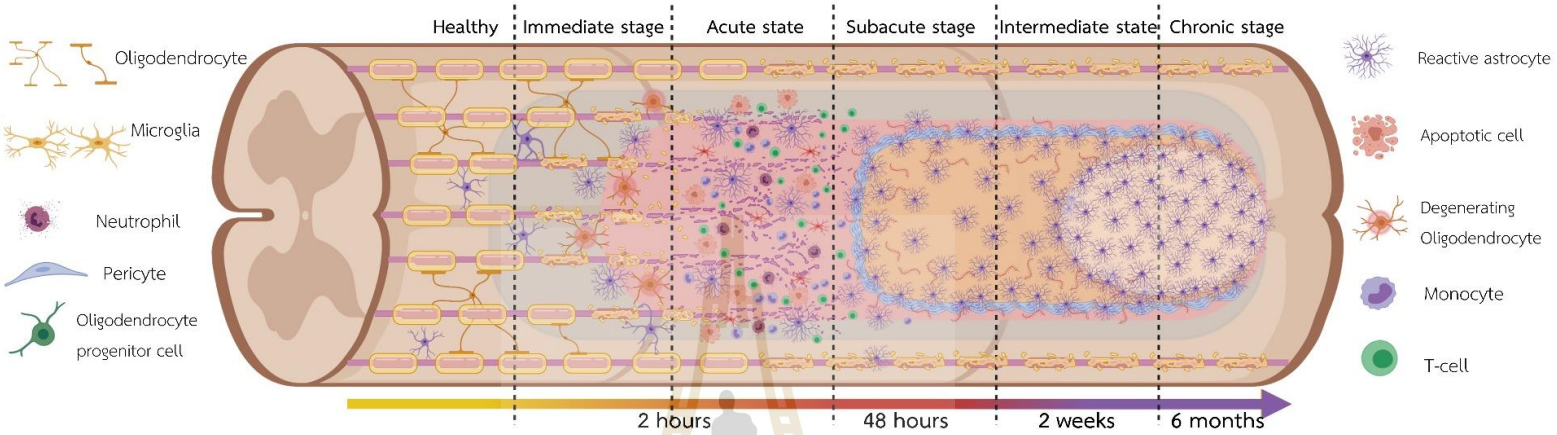

Figure 2.1 The pathophysiology of traumatic spinal cord injury. The initial mechanical trauma to the spinal cord initiates a secondary injury cascade that is characterized in the acute phase (0–48 hours after injury) by oedema, hemorrhage, ischemia, inflammatory cell infiltration, the release of cytotoxic products, and cell death. This secondary injury leads to necrosis and/or apoptosis of neurons and glial cells, such as oligodendrocytes, which can lead to demyelination and the loss of neural circuits. In the subacute phase (2-4 days after injury), further ischemia happens to be owed to ongoing oedema, vessel thrombosis, and vasospasm. Persistent inflammatory cell infiltration causes further cell death and cystic microcavities form as cells and the extracellular architecture of the cord are damaged. Furthermore, astrocytes proliferate and deposit extracellular matrix molecules into the perilesional area. In the intermediate and chronic phases, which last from two weeks to six months, axons continue to die off and the glial scar gets strong enough to stop regeneration. Cystic cavities unite to further restrict axonal regrowth and cell migration. Modification from Ahuja et al. (2017), created with BioRender.com

In brief, activation of glutamate receptors at high levels induces a rise in the influx of Na^{+} and $Ca2^{+}$ through N-Methyl-d-aspartic acid (NMDA) and alpha-amino-3-hydroxy-5-methyl-isoxazoleproprionate/kainate (AMPA) receptors, as well as excitotoxicity (Park et al., 2004). Moreover, FAS ligand receptors produced by microglia activation and p75 neurotrophin receptor signaling also activate the caspase and calpain cascades, which play a role in cellular apoptosis as well as responding to neuronal and glial death (Nagata and Golstein, 1995). Moreover, sensory inactivity and paralysis are caused by the loss of ionic channel homeostasis and neural cell death, together with demyelination signal transmission in the spinal cord (Rowland et al., 2008; Ruff and Fehlings, 2010; Tator and Fehlings, 1991). In addition, ROS is found 12 hours after SCI and continues to increase for nearly a week. Numerous ROS cause membrane lipid oxidation, cellular lysis, organelle destruction, and changes in $Ca2^{+}$ ion concentrations (Donnelly and Popovich, 2008; Park et al., 2004), which promotes the activation of calpains and caspase cascades (Schanne et al., 1979). The BBB, a highly selective endothelial filter for transporting the central nervous system, is the major reason for the mechanical damage and inflammation of spinal cord injury. The BBB induced an inflammatory response as well as an enhancement of astrocytes, T cells, neutrophils, microglia, and monocytes. In addition, the migration of macrophages and neutrophils causes cellular disruption and necrosis. For inflammatory noncellular mediators, TNF and IL-1 also promote vascular permeability. These inflammatory factors are caused by microglia and leukocytes, which are responsible for the failure of oxidative metabolism in demyelinated axons. Proliferation of astrocytes also increases the expression of glial fibrillary acid protein (GFAP) and the formation of glial or astrocytic scar structures (Rowland et al., 2008; Ruff and Fehlings, 2010; Wyndaele and Wyndaele, 2006). However, inhibition of some cytokines, such as TNF, has been demonstrated to result in functional neurological recovery after SCI and to have a neuroprotective effect *in vitro* and in animal models of SCI (Cheng et al., 1994; Kim et al., 2001).

3) Subacute stage

The subacute stage appears between two days and two weeks following the initial injury. During this stage, there is a gradual increase in the astrocytic intermediate filament due to the proliferative and hypertrophic activity of

astrocytes. These astrocytes function as a physical and chemical barrier to axonal regeneration by astrocytic scar or glial scar. However, in humans, there is significantly less glia scar than in rodent SCI models (Hagg and Oudega, 2006). Moreover, astrocytes support recovery by restoring ionic homeostasis and the integrity of the BBB, which reduces edema and immune cell invasion (Herrmann et al., 2008).

4) Intermediate stage

The intermediate stage begins two weeks after the accident and lasts for six months. During this stage, the glia scar matures and axonal regeneration begins (Salewski et al., 2013).

5) Chronic stage

Six months following the first SCI, the chronic phase would appear. The main characteristics of this stage, cysts, and the lesion are developed from glia scar formation and followed by Wallerian degeneration of severed and injured axons (Coleman and Perry, 2002; Hill et al., 2001; Rowland et al., 2008). Two to three years post-injury, the last stage of necrotic death is shown with cyst cavity formation and myelomalacia (McDonald et al., 2002). At this point, treatments are based on regenerative techniques that help damaged axons become more flexible, regenerate, and remyelinate.

2.4.2 Experimental models of spinal cord injury

The stages of damage are also used to classify SCI animal models. All SCI models are constructed in accordance with damage mechanisms, their characteristics, and their applicability to human SCI (Alizadeh et al., 2019).

1) Transection model

The transection model of SCI is very simple to generate. Nevertheless, this model is not representative of actual SCI or the clinical reality of SCI. Transection models are suitable for studying axonal regeneration or biomaterial scaffold surgery to close the gap between the proximal and distal portions of the severed spinal cord. There are two kinds of transection models: complete transection and partial transection models. Complete transection is the separation of the spinal cord from the higher motor centers, making it suited for the investigation of propriospinal motor and sensory circuits in the recovery of SCI locomotor (Barbeau et

al., 1999; Cheriyan et al., 2014). There are several sub-units of partial transection models such as hemisection, unilateral transection, and dorsal column lesions. Partial transection models are ideal for studying the pathophysiology of nerve grafts, plasticity, and comparisons between wounded and uninjured routes. However, these models do not exhibit significant injuries and have a high rate of spontaneous recovery. Thus, these models might not be appropriate for the development and assessment of novel medical treatments (Cheriyan et al., 2014).

2) Contusive model

Clinically, the contusion model is associated with SCI induced by acute physical trauma to the spinal cord. Animal models can now get contusions with three different kinds of equipment: the weight-drop apparatus, the computer-controlled impactor, and the air gun device.

The first contusion model impactor was produced by Gruner at New York University (NYU) in 1992 (Barbeau et al., 1999). In order to generate various phases of SCI, a metal rod of a certain weight (10 g) was dropped from different heights onto the spinal cord (Barbeau et al., 1999; Cheriyan et al., 2014). For analysis and verification, parameters such as time, impact velocity, and the biomechanical action of the tissue could be recorded. The NYU impactor or Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor, as well as modified protocols, are becoming standard for research. In 2012, the MASCIS impactor was modified to MACIS III. However, there are limitations to MASCIS impactors with control length of impact and weight bounce that could be caused by many impacts to predict the result of SCI (Cheriyan et al., 2014).

Another type is the Infinite Horizon (IH) impactor, which is produced by force-controlled impact. This model enhances the force management of impact and eliminates weight bouncing by using a computer-controlled metal impounder that may be rapidly retracted when dropped on the spinal cord. One of the benefits of the IH impactor is its ability to give rats with mild, moderate, and severe SCI by adjusting the force degree. However, the limitation of IH impactors is the uncontrolled firmness holding to the spinal column during the impact, which is a result of unpredictable injury and neurological deficits (Cheriyan et al., 2014).

The Ohio State University (OSU) impactor is another impactor produced to use a computer-controlled electromagnetic impactor, so multiple strikes are avoided (Cheriyan et al., 2014; Stokes, 1992). As a result of cerebrospinal fluid (CSF) displacement during device loading, the specific location of damage to the spinal cord cannot be confirmed with this impactor (Cheriyan et al., 2014). However, in 2000, the OSU impactor was developed for researchers in mice (Jakeman et al., 2000).

The last kind of contusion model is the air gun impactor, which was established by the rat model in 2012. High-pressure streams of air are used to produce SCI. The vertebrae are immovable. A dental drill is applied to the vertebral arch of the target vertebra to generate a two mm diameter hole. The injector is entered and placed between the holds without putting force on the dura mater. Then, the air shot is administered. The disadvantages of this technique are the instability of the specific injury quantification when the model is reproduced. Recently, MASCIS, IH, and OSU impactor devices produce SCI in small and big animals such as mice, rats, marmosets, cats, and pigs (Cheriyan et al., 2014; Gruner, 1992).

3) Compressive model

Compression injury consists of an initial contusion followed by a persistent compression caused by force application over a longer period of time (seconds to minutes). Consequently, the compression injury model may be suitable for contusive-compressive models (Cheriyan et al., 2014).

In 1978, the clip compression model was first presented, which is the most commonly used for SCI in rats and mice (Cheriyan et al., 2014; Joshi and Fehlings, 2002; Petteys et al., 2017). After laminectomy, a modified aneurism clip with a calibrated closure force is applied to the spinal cord for a predetermined amount of time (about one minute) to induce a contusive compressive injury (Cheriyan et al., 2014). The force of the clip and the duration of compression can be adjusted and modified to customize the stage of SCI injury. A fifty g clip for one minute, for example, can result in a severe SCI, whereas a thirty-five g clip for the same duration can result in a moderate to severe damage model (Poon et al., 2007). In recent years, smaller or bigger clips have been produced for usage in mouse (Joshi and Fehlings, 2002) and pig models (Lee et al., 2013). Comparison between clip compression and

contusion models, clip compression is less expensive and easier to working (Cheriyan et al., 2014). The clip compression model applies contusion and compression both dorsally and ventrally to the spinal cord, whereas a contusion is mainly applied dorsally. Since most SCIs in people are caused by burst compression fractures and dislocations, the clip compression model may be a better representation of this (Poon et al., 2007). However, it is not able to fully measure the closing velocity and delivered force at the moment of application due to a wide scope of variability (Cheriyan et al., 2014).

In 1991, Forceps compression, a straightforward and affordable compressive model, was originally used to induce SCI in guinea pigs (Blight, 1991). Although the spinal cord is compressed bilaterally using calibrated forceps with a spacer, this model still lacks the initial impact and contusive injury, which are associated with the majority of human traumatic SCI cases. As a result, the forceps compression model may not be a therapeutically relevant model for researching the pathology of human SCI and the development of new treatments (Cheriyan et al., 2014). Additionally, the balloon compression model has been widely used in primates and larger animals, such as dogs and cats (Aslan et al., 2009; Fukuda et al., 2005; Nesathurai et al., 2006).

4) Spinal cord strapping model

The developed mechanical device named SC-STRAPPER was used for a spinal cord strapping model for SCI induction. The suture is inserted into one side of an animal using a curved surgical needle. The suture is then wrapped around a certain level of the spinal cord in the epidural region and withdrawn through the opposite side of the skin. One end of the suture is attached to the wall of the experimental apparatus, while the other end is attached to a hanging mass by a simple pulley. The compression is started once the mass is permitted to drop for one minute. Mild, moderate, and severe injuries of SCI showed different functional and histological outcomes depending on the three different masses that were used. The advantages of the spinal cord strapping model are the comparatively noninvasive method, unrequired laminectomy, non-bleeding, and high percentage of survival was reported (da Costa et al., 2008). This model also delivers SCI not only in the dorsoventral or lateral direction compression but on all sides of the cord, and is able

to produce the injury grade. Nonetheless, the continuous reproducibility of injury at the exact location and range must be proved. Even though the compression force may be predicted based on the mass, other injury-related factors cannot be controlled or quantified (Cheriyan et al., 2014).

5) Chemical models

Chemical models were applied and applied to simulate the secondary injury caused by SCI. These models are appropriate for investigating the molecular processes of spinal cord injury and the effects of treatments on particular pathways. Reagents, such as superoxide, hydroxyl radical, and peroxynitrate, have been designed to construct reactive oxygen species, resulting in oxidative damage to lipids and proteins and therefore the degeneration of neurons. However, chemical models are hardly studied because of limitations such as wound growth and localization ability and non-repeatability of specific sites (Cheriyan et al., 2014).

In conclusion, although current animal models do not represent all clinical characteristics of human SCI. The compression and contusion models might be the best and most useful tools for studying secondary damage and developing therapies for SCI.

2.5 Neurorepair/neuroprotection after cell transplantation

The main mechanisms of neurorepair/neuroprotection after cell transplantation normally are trophic factor support, remyelination, and functional improvement (Nori et al., 2017). After cell transplantation therapy, neurorepair/neuroprotection function synergistically to promote plasticity and regeneration in SCI models. Neuroprotective mechanisms include the elimination of all endogenous toxins and inflammatory responses with or without the release of growth factors. After SCI model was transplanted by stem cells, neuroprotection might start with a mechanism of the acute and sub-acute stage of SCI. Next, transplanted cells may help the host by regrowing axons through the release of trophic factors, the decrease of chemicals that stop neurite growth, or remyelination, or by giving a wound an extracellular matrix to help it heal (Emgard et al., 2014).

Neurotrophic support is one of the important roles of neurorepair/ neuroprotection may be restored by cell transplantation after SCI. Neurotrophic factors secreted by stem cells to promote cell survival, cell differentiation, neuroprotection, remyelinating, axonal sparing, axonal growth, neuronal survival, synaptic plasticity with improved motor functional recovery play an important role in both the peripheral nervous system and central nervous system (Nori et al., 2017). Neurotrophic factors have been reported in neurorepair/neuroprotection for example brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), nerve growth factor (NGF), and neurotrophin-4/5 (NT-4/5) (Nori et al., 2017). In addition, cytokine growth factors such as the TGF-b family, GDNF, and hepatocyte growth factor (HGF) promote the development of axons following SCI (Nori et al., 2018). Cell-autonomous and non-cell-autonomous trophic effects have been shown in the study of NSs derived from human iPSC (adult human fibroblasts) after being transplanted to the SCI model (Nori et al., 2011). NSs induce the development of synapses between hiPSC-NS-derived neurons and mouse neurons. In addition, the activation of neurotrophic factors, angiogenesis, axonal regeneration in the distal spinal cord, and the number of myelin increased. hiPSC-NSs exhibited survival, migration, and differentiation into neurons, astrocytes, and oligodendrocytes in the injured spinal cord. The regeneration of axons by the transplanted hiPSC-NSs also demonstrated motor functional recovery.

In terms of cell differentiation, ESC-derived NSs were transplanted to both the acute and sub-acute phases of the SCI model (Silva, et al., 2017). One week and two weeks post-transplantation, transplanted cells differentiated into neural lineages including neurons and oligodendrocytes, as examined by specific markers including neural progenitor (NESTIN), oligodendrocyte progenitor cells (Olig2), neurons (MAP-2), astrocytes (GFAP), and oligodendrocytes (O4).

Myelin is an essential component of the central nervous system and is necessary for synapsing and communication. After SCI, extensive loss of white matter was shown, followed by the leak of functional axons. Remyelination was also detected after repair in SCI. In a rat model of chronic spinal cord injury, oligodendrocytes generated from human oligodendrogenic neural progenitor cells (oNPC) produced myelin sheaths and formed surviving axons. In addition,

$Kv1.2^{+}$/$Caspr^{+}$ nodes of Ranvier were found in graft-derived myelin sheaths, indicating the usefulness of grafted human oNPC-derived myelin sheaths (Nori et al., 2018).

The behavioral and functional effects of NSs treatment are most likely affected by a lot of factors. Functional improvement is often evaluated weekly by the Basso, Beattie, and Bresnahan (BBB) scale to study hindlimb locomotor recovery (Beattie et al., 1997). SCI model showed a promising score of 7.3 at the end of the 8 weeks after being transplanted with globose basal cells (GBCs) and NSs formation, along with electromyography amplitude, was markedly increased, indicating axonal regeneration (Muniswami et al., 2017). GBCs showed positive for β-tubulin III, neural cell marker, which could be responsible for the hindlimb motor recovery. However, there was a problem with tumor development after long-term post-transplantation of NSs, produced from iPSC. Transplantation of 253G1 (iPSC)-NSs to cure SCI in adult NOD-SCID mice resulted in three neural lineage differentiation, restoration of local circuitry, and stimulation of angiogenesis and axonal regeneration (Nori et al., 2015). Furthermore, they discovered that 253G1 (iPSC)-NS-grafted mice showed temporary motor function recovery through promoted synapse formation 47 days after transplantation, but a tumor formed for up to 103 days. Similar to transcriptome analysis, a higher mesenchymal transition may have helped the growth of tumors made from transplanted cells (Nori et al., 2015). Thus, the result of neurorepair/neuroprotection after transplantation depends on the selected various protocols for transplantation, such as origin of stem cells, induction protocol, SCI model, SCI stage etc. All of these factors lead to the effect of neurorepair/neuroprotection with a different outcome.

2.6 References

Ahuja, C. S., Wilson, J. R., Nori, S., Kotter, M. R. N., Druschel, C., Curt, A., & Fehlings, M. G. (2017). Traumatic spinal cord injury. **Nature Reviews Disease Primers**, *3*, 17018. doi:10.1038/nrdp.2017.18.

Alizadeh, A., Dyck, S. M., & Karimi-Abdolrezaee, S. (2019). Traumatic spinal cord injury: an overview of pathophysiology, models and acute injury mechanisms. **Frontiers in Neurology**, *10*, 282-282. doi:10.3389/fneur.2019.00282.

Aslan, A., Cemek, M., Eser, O., Altunbaş, K., Buyukokuroglu, M. E., Cosar, M., Baş, O., Ela, Y., & Fidan, H. (2009). Does dexmedetomidine reduce secondary damage after spinal cord injury? An experimental study. **European Spine Journal**, *18*(3), 336. doi:10.1007/s00586-008-0872-x.

Barbeau, H., McCrea, D. A., O'Donovan, M. J., Rossignol, S., Grill, W. M., & Lemay, M. A. (1999). Tapping into spinal circuits to restore motor function. **Brain Research Reviews**, *30*(1), 27-51. doi:10.1016/S0165-0173(99)00008-9.

Beattie, M. S., Bresnahan, J. C., Komon, J., Tovar, C. A., Van Meter, M., Anderson, D. K., Faden, A. I., Hsu, C. Y., Noble, L. J., Salzman, S., & Young, W. (1997). Endogenous repair after spinal cord contusion injuries in the rat. **Experimental Neurology**, *148*(2), 453-463. doi:10.1006/exnr.1997.6695.

Berry, M. F., Engler, A. J., Woo, Y. J., Pirolli, T. J., Bish, L. T., Jayasankar, V., Morine, K. J., Gardner, T. J., Discher, D. E., & Sweeney, H. L. (2006). Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. **American Journal of Physiology-Heart and Circulatory Physiology**, *290*(6), H2196-H2203. doi:10.1152/ajpheart.01017.2005.

Biernaskie, J., Sparling, J. S., Liu, J., Shannon, C. P., Plemel, J. R., Xie, Y., Miller, F. D., & Tetzlaff, W. (2007). Skin-derived precursors generate myelinating Schwann cells that promote remyelination and functional recovery after contusion spinal cord injury. **Journal of Neuroscience**, *27*(36), 9545-9559. doi:10.1523/JNEUROSCI.1930-07.2007.

Blight, A. R. (1991). Morphometric analysis of a model of spinal cord injury in guinea pigs, with behavioral evidence of delayed secondary pathology. **Journal of the Neurological Sciences**, *103*(2), 156-171. doi:10.1016/0022-510X(91)90159-5.

Bonnamain, V., Mathieux, E., Thinard, R., Thébault, P., Nerrière-Daguin, V., Lévêque, X., Anegon, I., Vanhove, B., Neveu, I., & Naveilhan, P. (2012). Expression of heme oxygenase-1 in neural stem/progenitor cells as a potential mechanism to evade host immune response. **Stem Cells**, *30*(10), 2342-2353. doi:10.1002/stem.1199.

Bonnamain, V., Neveu, I., & Naveilhan, P. (2011). *In vitro* analyses of the immunosuppressive properties of neural stem/progenitor cells using anti-cd3/cd28-activated t cells. In M. C. Cuturi & I. Anegon (Eds.), **Suppression and**

Regulation of Immune Responses: Methods and Protocols (pp. 233-243). Totowa, NJ: Humana Press.

Carlin, R., Davis, D., Weiss, M., Schultz, B., & Troyer, D. (2006). Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. **Reproductive Biology and Endocrinology : RB&E**, *4*, 8-8. doi:10.1186/1477-7827-4-8.

Cheng, B., Christakos, S., & Mattson, M. P. (1994). Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. **Neuron**, *12*(1), 139-153. doi:10.1016/0896-6273(94)90159-7.

Cheriyan, T., Ryan, D. J., Weinreb, J. H., Cheriyan, J., Paul, J. C., Lafage, V., Kirsch, T., & Errico, T. J. (2014). Spinal cord injury models: a review. **Spinal Cord**, *52*(8), 588-595. doi:10.1038/sc.2014.91.

Cizkova, D., Cubinkova, V., Smolek, T., Murgoci, A. N., Danko, J., Vdoviakova, K., Humenik, F., Cizek, M., Quanico, J., Fournier, I., & Salzet, M. (2018). Localized intrathecal delivery of mesenchymal stromal cells conditioned medium improves functional recovery in a rat model of spinal cord injury. **International Journal of Molecular Sciences**, *19*(3). doi:10.3390/ijms19030870.

Coleman, M. P., & Perry, V. H. (2002). Axon pathology in neurological disease: a neglected therapeutic target. **Trends in Neurosciences**, *25*(10), 532-537. doi:10.1016/s0166-2236(02)02255-5.

Condic, M. L. (2014). Totipotency: what it is and what it is not. **Stem Cells and Development**, *23*(8), 796-812. doi:10.1089/scd.2013.0364.

Cruz-Martinez, P., González-Granero, S., Molina-Navarro, M. M., Pacheco-Torres, J., García-Verdugo, J. M., Geijo-Barrientos, E., Jones, J., & Martinez, S. (2016). Intraventricular injections of mesenchymal stem cells activate endogenous functional remyelination in a chronic demyelinating murine model. **Cell Death and Disease**, *7*(5), e2223-e2223. doi:10.1038/cddis.2016.130.

da Costa, E. S. A., Carvalho, A. L., Martinez, A. M. B., De-Ary-Pires, B., Pires-Neto, M. A., & de Ary-Pires, R. (2008). Strapping the spinal cord: An innovative experimental

model of CNS injury in rats. **Journal of Neuroscience Methods**, *170*(1), 130-139. doi:10.1016/j.jneumeth.2008.01.004.

Desai, V. D., Hsia, H. C., & Schwarzbauer, J. E. (2014). Reversible modulation of myofibroblast differentiation in adipose-derived mesenchymal stem cells. **Plos One**, *9*(1), e86865-e86865. doi:10.1371/journal.pone.0086865.

Donnelly, D. J., & Popovich, P. G. (2008). Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. **Experimental Neurology**, *209*(2), 378-388. doi:10.1016/j.expneurol. 2007.06.009.

Emgard, M., Piao, J., Aineskog, H., Liu, J., Calzarossa, C., Odeberg, J., Holmberg, L., Samuelsson, E. B., Bezubik, B., Vincent, P. H., Falci, S. P., Seiger, A., Akesson, E., & Sundstrom, E. (2014). Neuroprotective effects of human spinal cord-derived neural precursor cells after transplantation to the injured spinal cord. **Experimental Neurology**, *253*, 138-145. doi:10.1016/j.expneurol.2013.12.022.

Fong, C. Y., Richards, M., Manasi, N., Biswas, A., & Bongso, A. (2007). Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. **Reproductive BioMedicine Online**, *15*(6), 708-718. doi: 10.1016/S1472-6483(10)60539-1.

Fukuda, S., Nakamura, T., Kishigami, Y., Endo, K., Azuma, T., Fujikawa, T., Tsutsumi, S., & Shimizu, Y. (2005). New canine spinal cord injury model free from laminectomy. **Brain Research Protocols**, *14*(3), 171-180. doi: 10.1016/j. brainresprot.2005.01.001.

Gardner, R. L. (2002). Stem cells: potency, plasticity and public perception. **Journal of Anatomy**, *200*(Pt 3), 277-282. doi:10.1046/j.1469-7580.2002.00029.x.

George, S., Hamblin, M. R., & Abrahamse, H. (2019). Differentiation of mesenchymal stem cells to neuroglia: in the context of cell signalling. **Stem Cell Reviews and Reports**, *15*(6), 814-826. doi:10.1007/s12015-019-09917-z.

Gruner, J. A. (1992). A monitored contusion model of spinal cord injury in the rat. **Journal of Neurotrauma**, *9*(2), 123-128. doi:10.1089/neu.1992.9.123.

Hagg, T., & Oudega, M. (2006). Degenerative and spontaneous regenerative processes after spinal cord injury. **Journal of Neurotrauma**, *23*, 264-280. doi:10.1089/ neu.2006.23.263.

Herrmann, J. E., Imura, T., Song, B., Qi, J., Ao, Y., Nguyen, T. K., Korsak, R. A., Takeda, K., Akira, S., & Sofroniew, M. V. (2008). STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. **Journal of Neuroscience**, *28*(28), 7231-7243. doi:10.1523/jneurosci.1709-08.2008.

Hill, C. E., Beattie, M. S., & Bresnahan, J. C. (2001). Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. **Experimental Neurology**, *171*(1), 153-169. doi:10.1006/exnr.2001.7734.

Hocking, A. M., & Gibran, N. S. (2010). Mesenchymal stem cells: Paracrine signaling and differentiation during cutaneous wound repair. **Experimental Cell Research**, *316*(14), 2213-2219. doi:10.1016/j.yexcr.2010.05.009.

Hosseini, S. M., Sani, M., Haider, K. H., Dorvash, M., Ziaee, S. M., Karimi, A., & Namavar, M. R. (2018). Concomitant use of mesenchymal stem cells and neural stem cells for treatment of spinal cord injury: A combo cell therapy approach. **Neuroscience Letters**, *668*, 138-146. doi:10.1016/j.neulet.2018.01.008.

Jakeman, L. B., Guan, Z., Wei, P., Ponnappan, R., Dzwonczyk, R., Popovich, P. G., & Stokes, B. T. (2000). Traumatic spinal cord injury produced by controlled contusion in mouse. **Journal of Neurotrauma**, *17*(4), 299-319. doi:10.1089/neu.2000.17.299.

Joshi, M., & Fehlings, M. G. (2002). Development and characterization of a novel, graded model of clip compressive spinal cord injury in the mouse: part 1. clip design, behavioral outcomes, and histopathology. **Journal of Neurotrauma**, *19*(2), 175-190. doi:10.1089/08977150252806947.

Jung, J., Kim, J.-W., Moon, H.-J., Hong, J. Y., & Hyun, J. K. (2016). Characterization of neurogenic potential of dental pulp stem cells cultured in xeno/serum-free condition: *in vitro* and *in vivo* assessment. **Stem Cells International**, *2016*, 6921097-6921097. doi:10.1155/2016/6921097.

Kakulas, B. A. (2004). Neuropathology: the foundation for new treatments in spinal cord injury. **Spinal Cord**, *42*(10), 549-563. doi:10.1038/sj.sc.3101670.

Katari, V., & Dwaipayan, S. (2017). Mesenchymal stem cells as a source of dopaminergic neurons: a potential cell based therapy for parkinson disease.

Current Stem Cell Research & Therapy, *12*(4), 326-347. doi: 10.2174/1574888X12666161114122059.

Kawase-Koga, Y., Fujii, Y., Yamakawa, D., Sato, M., & Chikazu, D. (2020). Identification of neurospheres generated from human dental pulp stem cells in xeno-/serum-free conditions. **Regenerative Therapy**, *14*, 128-135. doi:10.1016/j.reth. 2019.11.006.

Kim, G. M., Xu, J., Xu, J., Song, S. K., Yan, P., Ku, G., Xu, X. M., & Hsu, C. Y. (2001). Tumor necrosis factor receptor deletion reduces nuclear factor-kappaB activation, cellular inhibitor of apoptosis protein 2 expression, and functional recovery after traumatic spinal cord injury. **Journal of Neuroscience**, *21*(17), 6617-6625. doi:10.1523/jneurosci.21-17-06617.2001.

Lee, J. H. T., Jones, C. F., Okon, E. B., Anderson, L., Tigchelaar, S., Kooner, P., Godbey, T., Chua, B., Gray, G., Hildebrandt, R., Cripton, P., Tetzlaff, W., & Kwon, B. K. (2013). A Novel porcine model of traumatic thoracic spinal cord injury. **Journal of Neurotrauma**, *30*(3), 142-159. doi:10.1089/neu.2012.2386.

Li, H., Radford, J. C., Ragusa, M. J., Shea, K. L., McKercher, S. R., Zaremba, J. D., Soussou, W., Nie, Z., Kang, Y. J., Nakanishi, N., Okamoto, S., Roberts, A. J., Schwarz, J. J., & Lipton, S. A. (2008). Transcription factor MEF2C influences neural stem/progenitor cell differentiation and maturation *in vivo*. **Proceedings of the National Academy of Sciences of the United States of America**, *105*(27), 9397-9402. doi:10.1073/pnas.0802876105.

Li, Z., Zhou, G., Jiang, L., Xiang, H., & Cao, Y. (2018). Effect of STOX1 on recurrent spontaneous abortion by regulating trophoblast cell proliferation and migration via the PI3K/AKT signaling pathway. **Journal of Cellular Biochemistry**, *120*(5), 8291-8299. doi:10.1002/jcb.28112.

Lipton, S. A., & Rosenberg, P. A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. **New England Journal of Medicine**, *330*(9), 613-622. doi:10.1056/nejm199403033300907.

Loris, Z. B., Pieper, A. A., & Dietrich, W. D. (2017). The neuroprotective compound P7C3-A20 promotes neurogenesis and improves cognitive function after ischemic stroke. **Experimental Neurology**, *290*, 63-73. doi:10.1016/j. expneurol.2017.01.006.

McDonald, J. W., Becker, D., Sadowsky, C. L., Jane, J. A., Sr., Conturo, T. E., & Schultz, L. M. (2002). Late recovery following spinal cord injury. Case report and review of the literature. **Journal of Neurosurgery**, *97*(2 Suppl), 252-265. doi:10.3171/spi.2002.97.2.0252.

Miguel, M. P. D., Fuentes-Julian, S., Blazquez-Martinez, A., Pascual, C. Y., Aller, M. A., Arias, J., & Arnalich-Montiel, F. (2012). Immunosuppressive Properties of Mesenchymal Stem Cells: Advances and Applications. **Current Molecular Medicine**, *12*(5), 574-591. doi:10.2174/156652412800619950.

Mitalipov, S., & Wolf, D. (2009). Totipotency, pluripotency and nuclear reprogramming. **Advances In Biochemical Engineering/Biotechnology**, *114*, 185-199. doi:10.1007/10_2008_45.

Mouhieddine, T. H., Kobeissy, F. H., Itani, M., Nokkari, A., & Wang, K. K. W. (2014). Stem cells in neuroinjury and neurodegenerative disorders: challenges and future neurotherapeutic prospects. **Neural Regeneration Research**, *9*(9), 901-906. doi:10.4103/1673-5374.133129.

Muniswami, D. M., Kanakasabapathy, I., & Tharion, G. (2017). Globose basal cells for spinal cord regeneration. **Neural Regeneration Research**, *12*(11), 1895-1904. doi:10.4103/1673-5374.219052.

Nagata, S., & Golstein, P. (1995). The Fas death factor. **Science**, *267*(5203), 1449-1456. doi:10.1126/science.7533326.

Nesathurai, S., Graham, W. A., Mansfield, K., Magill, D., Sehgal, P., Westmoreland, S. V., Prusty, S., Rosene, D. L., & Sledge, J. B. (2006). Model of traumatic spinal cord injury in Macaca fascicularis: similarity of experimental lesions created by epidural catheter to human spinal cord injury. **Journal of Medical Primatology**, *35*(6), 401-404. doi:10.1111/j.1600-0684.2006.00162.x.

Nori, S., Khazaei, M., Ahuja, C. S., Yokota, K., Ahlfors, J.-E., Liu, Y., Wang, J., Shibata, S., Chio, J., Hettiaratchi, M. H., Führmann, T., Shoichet, M. S., & Fehlings, M. G. (2018). Human oligodendrogenic neural progenitor cells delivered with chondroitinase ABC facilitate functional repair of chronic spinal cord injury. **Stem Cell Reports**, *11*(6), 1433-1448. doi:10.1016/j.stemcr.2018.10.017.

Nori, S., Nakamura, M., & Okano, H. (2017). Chapter 2 - Plasticity and regeneration in the injured spinal cord after cell transplantation therapy. In S. B. Dunnett & A. Björklund (Eds.), **Progress in Brain Research** (Vol. 231, pp. 33-56): Elsevier.

Nori, S., Okada, Y., Nishimura, S., Sasaki, T., Itakura, G., Kobayashi, Y., Renault-Mihara, F., Shimizu, A., Koya, I., Yoshida, R., Kudoh, J., Koike, M., Uchiyama, Y., Ikeda, E., Toyama, Y., Nakamura, M., & Okano, H. (2015). Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: oncogenic transformation with epithelial-mesenchymal transition. **Stem Cell Reports**, *4*(3), 360-373. doi:10.1016/j.stemcr.2015.01.006.

Nori, S., Okada, Y., Yasuda, A., Tsuji, O., Takahashi, Y., Kobayashi, Y., Fujiyoshi, K., Koike, M., Uchiyama, Y., Ikeda, E., Toyama, Y., Yamanaka, S., Nakamura, M., & Okano, H. (2011). Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. **Proceedings of the National Academy of Sciences of the United States of America**, *108*(40), 16825-16830. doi:10.1073/pnas.1108077108.

Park, E., Velumian, A. A., & Fehlings, M. G. (2004). The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. **Journal of Neurotrauma**, *21*(6), 754-774. doi:10.1089/0897715041269641.

Peng, C., Li, Y., Lu, L., Zhu, J., Li, H., & Hu, J. (2019). Efficient one-step induction of human umbilical cord-derived mesenchymal stem cells (UC-MSCs) produces msc-derived neurospheres (MSC-NS) with unique transcriptional profile and enhanced neurogenic and angiogenic secretomes. **Stem Cells International**, *2019*, 9208173. doi:10.1155/2019/9208173.

Petrik, D., Jiang, Y., Birnbaum, S. G., Powell, C. M., Kim, M.-S., Hsieh, J., & Eisch, A. J. (2012). Functional and mechanistic exploration of an adult neurogenesis-promoting small molecule. **FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology**, *26*(8), 3148-3162. doi:10.1096/fj.11-201426.

Petteys, R. J., Spitz, S. M., Syed, H., Rice, R. A., Sarabia-Estrada, R., Goodwin, C. R., Sciubba, D. M., & Freedman, B. A. (2017). Design and testing of a controlled electromagnetic spinal cord impactor for use in large animal models of acute

traumatic spinal cord injury. **Journal of Clinical Neuroscience**, *43*, 229-234. doi:10.1016/j.jocn.2017.04.031.

Pineau, I., & Lacroix, S. (2007). Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. **Journal of Comparative Neurology**, *500*(2), 267-285. doi:10.1002/cne.21149.

Poon, P. C., Gupta, D., Shoichet, M. S., & Tator, C. H. (2007). Clip compression model is useful for thoracic spinal cord injuries: histologic and functional correlates. **Spine**, *32*(25).

Potthoff, M. J., & Olson, E. N. (2007). MEF2: a central regulator of diverse developmental programs. **Development**, *134*(23), 4131-4140. doi:10.1242/dev.008367.

Ranjbaran, H., Abediankenari, S., Mohammadi, M., Jafari, N., Khalilian, A., Rahmani, Z., Amiri, M., & Ebrahimi, P. (2018). Wharton's jelly derived-mesenchymal stem cells: isolation and characterization. **Acta medica Iranica**, *56*, 28-33.

Rowland, J. W., Hawryluk, G. W., Kwon, B., & Fehlings, M. G. (2008). Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. **Neurosurgical Focus**, *25*(5), E2. doi:10.3171/foc.2008.25.11.E2.

Ruff, C. A., & Fehlings, M. G. (2010). Neural stem cells in regenerative medicine: bridging the gap. **Panminerva Medica**, *52*(2), 125-147.

Salewski, R., Emrani, H., & G, M. (2013). Neural Stem/Progenitor Cells for Spinal Cord Regeneration. In: InTech.

Schanne, F. A., Kane, A. B., Young, E. E., & Farber, J. L. (1979). Calcium dependence of toxic cell death: a final common pathway. **Science**, *206*(4419), 700-702. doi:10.1126/science.386513.

Schneider, J. W., Gao, Z., Li, S., Farooqi, M., Tang, T. S., Bezprozvanny, I., Frantz, D. E., & Hsieh, J. (2008). Small-molecule activation of neuronal cell fate. **Nature Chemical Biology**, *4*(7), 408-410. doi:10.1038/nchembio.95.

Silva, J., Bento, A. R., Barros, D., Laundos, T. L., Sousa, S. R., Quelhas, P., Sousa, M. M., Pego, A. P., & Amaral, I. F. (2017). Fibrin functionalization with synthetic adhesive ligands interacting with alpha6beta1 integrin receptor enhance

neurite outgrowth of embryonic stem cell-derived neural stem/progenitors. **Acta Biomaterialia**, *59*, 243-256. doi:10.1016/j.actbio.2017.07.013.

Silva, J., Bento, A. R., Barros, D., Laundos, T. L., Sousa, S. R., Quelhas, P., Sousa, M. M., Pêgo, A. P., & Amaral, I. F. (2017). Fibrin functionalization with synthetic adhesive ligands interacting with **α6β1** integrin receptor enhance neurite outgrowth of embryonic stem cell-derived neural stem/progenitors. **Acta Biomaterialia**, *59*, 243-256. doi:10.1016/j.actbio.2017.07.013.

Sontag, C. J., Uchida, N., Cummings, B. J., & Anderson, A. J. (2014). Injury to the spinal cord niche alters the engraftment dynamics of human neural stem cells. **Stem Cell Reports**, *2*(5), 620-632. doi:10.1016/j.stemcr.2014.03.005.

Stokes, B. T. (1992). Experimental Spinal cord injury: a dynamic and verifiable injury device. **Journal of Neurotrauma**, *9*(2), 129-134. doi:10.1089/neu.1992.9.129.

Tator, C. H., & Fehlings, M. G. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. **Journal of Neurosurgery**, *75*(1), 15-26. doi:10.3171/jns.1991.75.1.0015.

Tator, C. H., & Koyanagi, I. (1997). Vascular mechanisms in the pathophysiology of human spinal cord injury. **Journal of Neurosurgery**, *86*(3), 483-492. doi:10.3171/jns.1997.86.3.0483.

Wang, G., Han, T., Nijhawan, D., Theodoropoulos, P., Naidoo, J., Yadavalli, S., Mirzaei, H., Pieper, A. A., Ready, J. M., & McKnight, S. L. (2014). P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. **Cell**, *158*(6), 1324-1334. doi:10.1016/j.cell.2014.07.040.

Wang, Y. H., Liou, K. T., Tsai, K. C., Liu, H. K., Yang, L. M., Chern, C. M., & Shen, Y. C. (2018). GSK-3 inhibition through GLP-1R allosteric activation mediates the neurogenesis promoting effect of P7C3 after cerebral ischemic/reperfusional injury in mice. **Toxicology and Applied Pharmacologyl**, *357*, 88-105. doi:10.1016/j.taap.2018.08.023.

Wu, Y., Chen, L., Scott, P. G., & Tredget, E. E. (2007). Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. **Stem Cells**, *25*(10), 2648-2659. doi:10.1634/stemcells.2007-0226.

Wyndaele, M., & Wyndaele, J. J. (2006). Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? **Spinal Cord**, *44*(9), 523-529. doi:10.1038/sj.sc.3101893.

CHAPTER III

TRANSDIFFERENTIATION OF HUMAN WHARTON'S JELLY DERIVED-MESENCHYMAL STEM CELLS TO BE NEUROSPHERE

3.1 Abstract

MSCs are multipotent, self-renewing stromal cells that can differentiate into a variety of cell types. MSCs have increased proliferative potential, decreased inflammation, and no tumorigenic possibilities. These cells are capable of inducing tissue regeneration and preventing the development of malignancies following stem cell transplantation. hWJ-MSCs have been isolated and characterized and then induced in vitro to create NSs suspended in serum-free medium. Small molecules P7C3-20 and Isx9 were used to stimulate and promote neuron differentiation performance with hWJ-MSCs. The results of immunocytochemistry and gene expression showed hWJ-MSCs could differentiate into NSs using media supplemented with 10 uM Isx9. All results showed NSs expressed neural stem/progenitor cell markers via the Wnt3A signaling pathway. Thus, NSs from day 7 of the Isx9 group were used for further NS cell transplantation in rats.

3.2 Introduction

MSCs are multipotent, self-renewing stromal cells that may develop into several cell types. MSCs are excellent options for cell therapy since they are freely available, simple to deliver, have a high rate of survival after cryopreservation, and they have not shown any negative impacts in allogeneic transplantation (Malgieri et al., 2010). Previous studies have shown that MSCs can regulate the growing microenvironment of NSCs and promote their survival rate (Zhou et al., 2016). These cells have enhanced proliferative capacity, decreased inflammation, and no tumorigenesis (Kim et al, 2013; Zhou et al., 2016). MSCs release large amounts of NGF, neurotrophic factors NT-3, NT-4, bFF, and GDNF, as well as other neuroprotective compounds, in addition to stimulating neurogenesis and

angiogenesis (Balasubramanian et al., 2013). Significantly, MSCs could induce tissue regeneration and prevent the development of tumors associated with stem cell transplantation (Sun et al, 2019). MSCs are present in a variety of tissues, including the umbilical cord, bone marrow, placenta, and adipose tissue (Venkatesh and Sen, 2017). Consequently, the therapeutic value of MSCs isolated from umbilical cord Wharton's jelly has developed (Balasubramanian et al., 2013).

MSCs are generated *in vitro* to produce NSs suspended in serum-free media (George et al., 2019). NSs are a hybrid of NSCs and NPCs, as NSPCs are (Kawase-Koga et al., 2020). NSs are multipotent stem cells with the capacity for self-renewal and differentiation into neural lineages, neurons, astrocytes, and oligodendrocytes. It is interesting that no HLA-DR expression was identified in these NSs, making them a suitable opportunity for transplantation treatment (Pen et al., 2019). According to a recent study, UC-MSCs can be processed to produce neurons under conventional culture conditions (DMEM/F12, EGF, bFGF, N2, and B27) after 12 hours (Peng et al., 2019). Moreover, P7C3-20 and Ix9 small molecules exhibit high neuron induction performance (Yang et al., 2019). The addition of the small molecule P7C3-A20 improved the efficiency of spinal cord-NSC neuronal development significantly (Yang et al., 2021). The Isx9 small molecule also highly stimulated neuronal development in adult neural stem cells by rapidly signaling the neuronal lineages *in vitro* via $Ca2^{+}$ influx (Schneider et al., 2008; Shohayeb et al., 2018). In this study, P7C3-20 and Isx9 small molecules might be useful to differentiate hWJ-MSCs via stimulating and promoting neurogenesis. This procedure might be applicable for neurodegenerative disease transplantation treatment.

3.3 Materials and methods

3.3.1 Ethics Statement

This study was approved by the Ethics Committee for Research Involving Human Subjects, Suranaree University of Technology (EC-61-58). Human umbilical cord tissues were harvested and preserved aseptically after delivery with informed consent from patients at Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima.

3.3.2 Reagents

Chemicals used in this research were from Sigma-Aldrich Corporation (St. Louis, MO, USA), antibodies were from Merck Ltd. (Darmstadt, Germany), the cell culture media were from Gibco (Paisley, UK), and plastic cell culture devices were from SPL life sciences (Gyeonggi-do, South Korea), if not, specify additionally.

3.3.3 hWJ-MSCs isolation and culture

Two samples of human umbilical cord were isolated. Human umbilical cords around 7-10 cm long were cleaned and both sides were cut off. Affer that, umbilical cord vessels were separated. The gelatinous tissue surrounding the vessels were collected and chopped into small pieces (3 mm^2) and were plated on sterile 90x15 mm culture dishes and left for 3-5 min at room temperature (RT) to enable tissue to attach to the dish. MSCs culture medium (alpha modification of Eagle's medium (α-MEM) supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin) was carefully added to the culture dish. Tissues were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C for 10-14 days. The medium was changed every 3 days. MSCs were expanded until passage 3 and either directly used for experiments or cryopreserved with 10% dimethyl sulfoxide (DMSO) and kept in liquid nitrogen.

3.3.4 Flow cytometric analysis of hWJ-MSCs

Flow cytometric analysis was performed to confirm MSCs surface markers. Two cell lines of hW1-MSCs were performed. The adherent MSCs cells were treated with trypsin-EDTA and collected after being centrifuged at 350xg for 5 min. MSCs were incubated in antibodies in the dark for 20 min. All antibodies are listed in appendix table 1. Each antibody was diluted in 100 ml of PBS(-), following antibodies: anti-CD34-PE (dilution 1:10, Beckman Coulter, Brea, California, USA), anti-CD45-FITC (dilution 1:20, Biolegend, San Diego, California, USA), anti-CD73-APC, anti-CD90-APC/A750, and anti-CD105-PE (dilution 1:100, Biolegend). The samples were analyzed by an Attune™ NxT Flow Cytometer (Attune™ NXT, Thermo Fisher Scientific, Cleveland, OH, USA). The percentage of $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, and $CD105^+$ positive or negative cell populations were calculated using the FCS Express™ Software.

3.3.5 Trilineage differentiation ability of hWJ-MSCs

As described prevously (Panta et al, 2019) MSCs at passage 5 of two cel in were cultured until 70% confluence in 6-well culture plates coated with 0.1% gelatin. To induce osteogenic lineage, the induction medium was α-MEM medium supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, 10 mM β-glycerophosphate, 100 units/ml penicillin, and 100 µg/ml streptomycin. The induction medium was replaced every 2 days and cultured for 21 days. Calcium deposits from the cells were stained and visualized by alizarin red staining.

To induce adipogenic lineage, MSCs were cultured in a-MEM medium supplemented with 5% FBS, 10 µg/ml insulin, 100 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutyl methylxanthine (IBMX), 100 units/ml penicillin and 100 µg/ml streptomycin. IBMX was removed from the medium after 1 week of induction. The medium was replaced every 2 days and cultured for 21 days. Cells were stained with Oil Red O to observe oil droplets.

To induce chondrogenic lineage, MSCs were cultured in a-MEM medium supplemented with 1% FBS, 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X), 50 µg/ml ascorbate-2-phosphate, 40 µg/ml L-proline, 100 µg/ml sodium pyruvate, 100 nM dexamethasone, 10 ng/ml of transforming growth factor beta-3 (TGFβ-3), 100 units/ml penicilin and 100 µg/ml streptomycin. The medium was replaced every 2 days and cultured for 21 days. Glycosaminoglycans (GAG) production was assessed by Alcian blue 8x staining.

3.3.6 Analysis of P7C3-A20 and Isx9 cytotoxicity

One thousand MSCs were cultured in 96-well culture plates in MSCs culture medium for 6h to allow cell attachment. Neurogenesis enhancing small molecules, Isx9 and P7C3-A20 (MedChemExpress, NJ, USA) cytotoxicity were evaluated by addition into the culture medium at 0, 1, 2.5, 5, 10 and 20 pM for Isx9 and at 0, 0.25, 0.50, 1, 2.5 and 5 µM for P7C3-A20. The cells were cultured at 37°C for 48h in a humidified atmosphere of 5% CO_2. The cytotoxicity of Isx9 and P7C3-A20 on cell viability was analyzed by a 3 (4,5-dimethytthiazol-2-y/) 2,5-diphenyltetrazolium bromide (MTT; Invitrogen™, Thermo Fisher Scientific, Massachusetts, USA) assay followed the manufacturer's instructions.

3.3.7 Neurosphere differentiation

MSCs cells passage 6 were seeded in 60x15 mm petri dish (Greiner Bio-One, Kremsmünster, Austria). Approximately $2.4x10^6$ cells were resuspend in neurosphere culture medium; NM (Bonilla-Porras et al., 2017). NM was composed of DMEM/F-12 medium supplemented with 2% B27, 1% N2, 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 2 µg/ml heparin sodium salt, 100 units/ml penicillin and 100 µg/mL streptomycin combined with or without 3 µM P7C3 and/or 10 µM Isx9. Cells were cultured in a humidified atmosphere at 37°C and 5% CO_2 for 14 days. Twenty-four hours after culture, the medium replaced every 3 days. The experimental groups were divided into 3, 7, 10, and 14 days after induction (Fig. 3.1), (1) control; cultured with DMEM/F12 (1:1), (2) P7C3; cultured with NM+3 µM P7C3, (3) Isx9; cultured with NM+10 µM Isx9, (4) P7C3+Isx9; cultured with NM+10 µM Isx9 for 3 days then changed to NM+10 µM Isx9+3 µM P7C3 until 14 days.

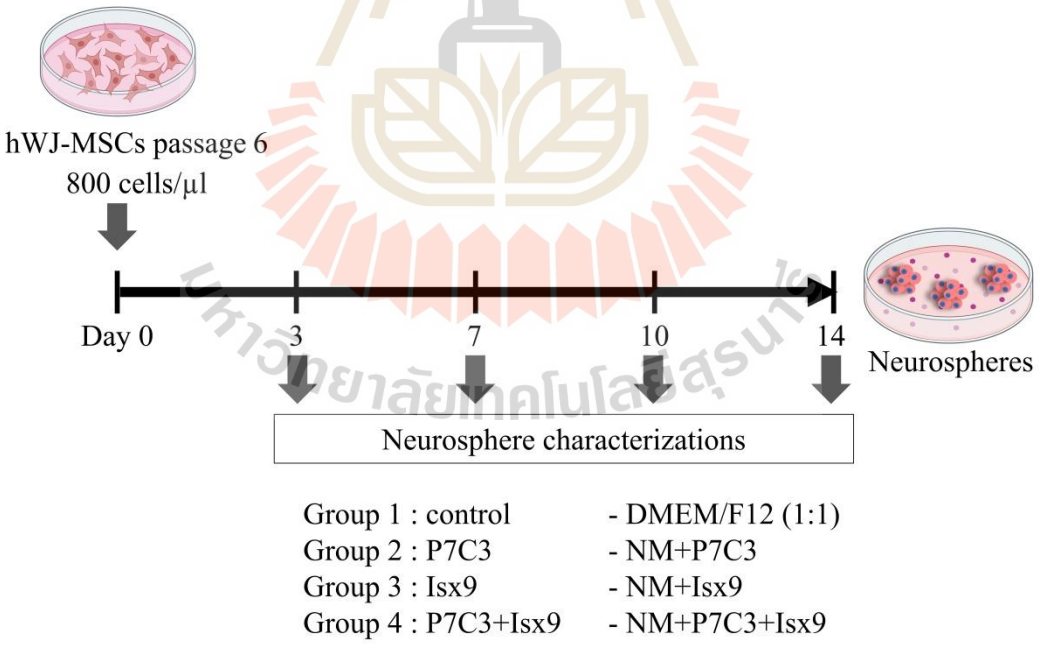

Figure 3.1 Neurosphere differentiation experimental design.

3.3.8 Neurosphere characterizations

1) Immunocytochemical staining

Neurospheres of day 3, 7, 10, and 14 were filtered with nylon net filters (#NY1H02500 (100μm) and #NY4H04700 (140μm), Merck Millipore, Massachusetts, USA), and only 100-140 pm in diameter were selected (Nori et al., 2015). The cells were analyzed for the neural stem/progenitor cell expression by immunocytochemical staining (ICC). Start with, neurospheres were fixed with 100% methanol for 20 min at -20℃, permeabilized, and blocked with 2% bovine serum albumin (BSA), 5% normal goat serum, 3 mM sodium azide, and 0.2% triton-X-100. All antibodies are listed in appendix table 1. Cells were incubated for 2h at RT following by incubation with primary antibodies; anti-nestin antibody (dilution 1:100; neural stem/progenitor cell marker), anti-SOX2 antibody (dilution 1:100, Bioscience, Massachusetts, USA; neural stem/progenitor cell marker), anti-β-tubulin 3 antibody (dilution 1:100; neuron cell marker), anti- β-catenin antibody (dilution 1:100; Wnt3A signaling pathway marker), and anti-DCX antibody (dilution 1:100; immature neuron cells marker) overnight at 4℃. Next day, neurospheres were incubated in secondary antibodies; Alexa fluor® 488 donkey anti-mouse IgG (dilution 1:1000, Invitrogen™, Thermo Fisher Scientific), Alexa fluor® 594 goat anti-rat 1gG (dilution 1:250, Invitrogen™, Thermo Fisher Scientific) and Alexa fluor® 594 goat anti-rabbit 1gG (dilution 1:250, Invitrogen™, Thermo Fisher Scientific) for 2h at RT. Then, cells were stained with 6-diamino-2-phenylindole (DAPI; 1:1000) and mounted with Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). Samples were observed using a fluorescence inverted microscope (Eclipse Ti-S, Nikon Imaging Japan Inc.) by the NIS-Elements D program (Nikon Imaging Japan Inc., Tokyo, Japan).

2) Gene expression analysis

To determine mRNA expression of neurospheres, total RNA was isolated after 3, 7, 10, and 14 days of differentiation, using FavorPrep Tissue Total RNA Mini Kit (Favorgen Biotech Corp., PingTung, Taiwan) followed the manufacturer's instructions. The, RNAs were then reverse-transcribed using oligo-dT primers for cDNA synthesis (biotech rabbit GmbH, Berlin, Germany). Neurosphere-specific gene expressions were evaluated using KAPA SYBR FAST qPCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Gene expression was examined with QuantStudio 5 real-time PCR system (QuantStudio 5, Applied Biosystems, Carlsbad, CA, USA). Melting

curve analysis was also performed to specificity of the specific primers (Table 3.1). *β-actin* was used as a reference gene to normalized the target genes the expression fold change was calculated relative to control cells, MSCs, and positive control, SHSY-5Y human neuroblastoma (ATCC #CRL-2266). qPCR was performed in triplicate and statistical analysis was performed using the $2^{-\Delta\Delta CT}$ method.

Table 3.1 Primers used for qPCR.

Genes	Primer sequence (5'→3')	Product size (bp)	References
β-catenin	F: CTGAGGACAAGCCACAAGATTACA R: TGGGCACCAATATCAAGTCCAA	121	NM 001330729.2
β-tubulin 3	F: TGGATCCCCAACAACGTGAA R: CITCGTACATCTCCCCTCTT	286	NM 006086.4
NEUROD1	F: TCTTCCACGTTAAGCCTCCG R: CCATCAAAGGAAGGGCTGGT	97	NM 002500.5
SOX2	F: GCGGAAAACCAAGACGCTC R: TTCATGTGCGCGTAACTGTC	153	BC 013923.2
nestin	F: AGTGATGCCCCTTCACCTTG R: GCTCGCTCTCTACTITCCCC	199	NM 006617.1
DCX	F: TATGCGCCGAAGCAAGTCTCCA R: CATCCAAGGACAGAGGCAGGTA	155	NM 000555.3
*β-actin**	F: GAGAAAATCTGGCACCACACC R: GGATAGCACAGCCTGGATAGCAA	177	NM 001101.5

*Reference gene

3) Western blotting analysis

To determine the level of protein expression for Wnt3A signaling pathway markers and neural stem/progenitor cell markers, extracted protein of neurospheres at day 0, 3, 7, 10, and 14 of differentiation were separated via SDS-PAGE (7.5% or 10% resolving gel), followed by electro-transfer to PDF membranes (Immun-Blot® PVDF Membrane, Bio-Rad Laboratories, Hercules, CA, USA). Afterward, the membranes were blocked with 5% skim milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1h at RT. For protein detection of nestin, β-tubulin 3, NEUROD1, SOX2, β-catenin, and DCX protein membranes were incubated with primary antibody solution

(1% BSA in TBS, Tris-buffered saline) overnight at 4°C. After washed by TBST, membranes were incubated for 1h at RT with secondary antibodies conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, UK), diluted 1:2000 in 5% skim milk in TBST, apply the chemiluminescent substrate by using ECL substrate kit (Ultra-high sensitivity, Abcam, Cambridge, UK) following the manufacturer's suggestions. Protein bands were imaged by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Massachusetts, USA) and then quantified using Image J software (National Institute of Health, Maryland, USA). All antibodies are listed in appendix table 1. β-actin was used as housekeeping control for normalization with nestin, β-tubulin 3, NEUROD1, SOX2, B-catenin, and DCX protein. The data were compared to negative control cells (MSCs) and positive control (SHSY-5Y) and no data were quantified from bands with saturated pixels.

3.3.9 Statistical Analysis

Statistical analysis was performed using GraphPad version 5 and data were expressed as the mean±S.D. The differences between values were determined using a one-way analysis of variance (ANOVA), followed by Tukey-Kramer Honest Significant Difference (HSD) Post hoc test to compare differences between two groups. A value of $p<0.05$ was considered sienificant with different lowercase letters. The data was plotted by GraphPad Prism version 5.

3.4 Result

3.4.1 MSCs characterization

Characteristics of MSCs including morphology, surface marker expression, and differentiation potential were determined. After hWJ-MSCs isolation for 10-14 days, cells were expanded and passaged. At passage 5, cell morphology of both cell lines was similar to monolayer fibroblast cells (Fig. 3.2A). Cell surface markers expression of MSCs were analyzed by flow cytometry (Fig. 3.3). Cell line 1, the results were positive for CD73 (99.51%), CD90 (99.77%), and CD105 (99.72%) cell surface markers and negative for CD34 (100.00%) and CD45 (99.99%) cell surface markers. But cell line 2, the results were positive for CD73 (99.79%), CD90 (99.60%), and CD105 (2.42%) cell surface markers and negative for CD34 (100.00%) and CD45 (99.99%) cell surface markers.

Differentiation of MSCs to osteocyte, chondrocyte, and adipocyte cells were done by using induction medium for 21 days (Fig. 3.2B). Only MSCs from cell line 1 could

differentiate to be all osteocyte, chondrocyte, and adipocyte cells. Osteocyte differentiation results of cell line 1 showed cells with intracellular calcium accumulation and stained with Alizarin red. Adipocyte differentiation results of cell line 1 showed the intracellular lipids (lipid droplets) in cells were found and stained by oil red O dye. For chondrocyte differentiation of both cell line could differentiate into chondrocytes as tested by the presence of Alcian blue-stained region.

Thus, cell line 1 was accepted for MSCs characterization standards from the International Society for Cell & Gene Therapy, therefore this cell line was then used in further experiments.

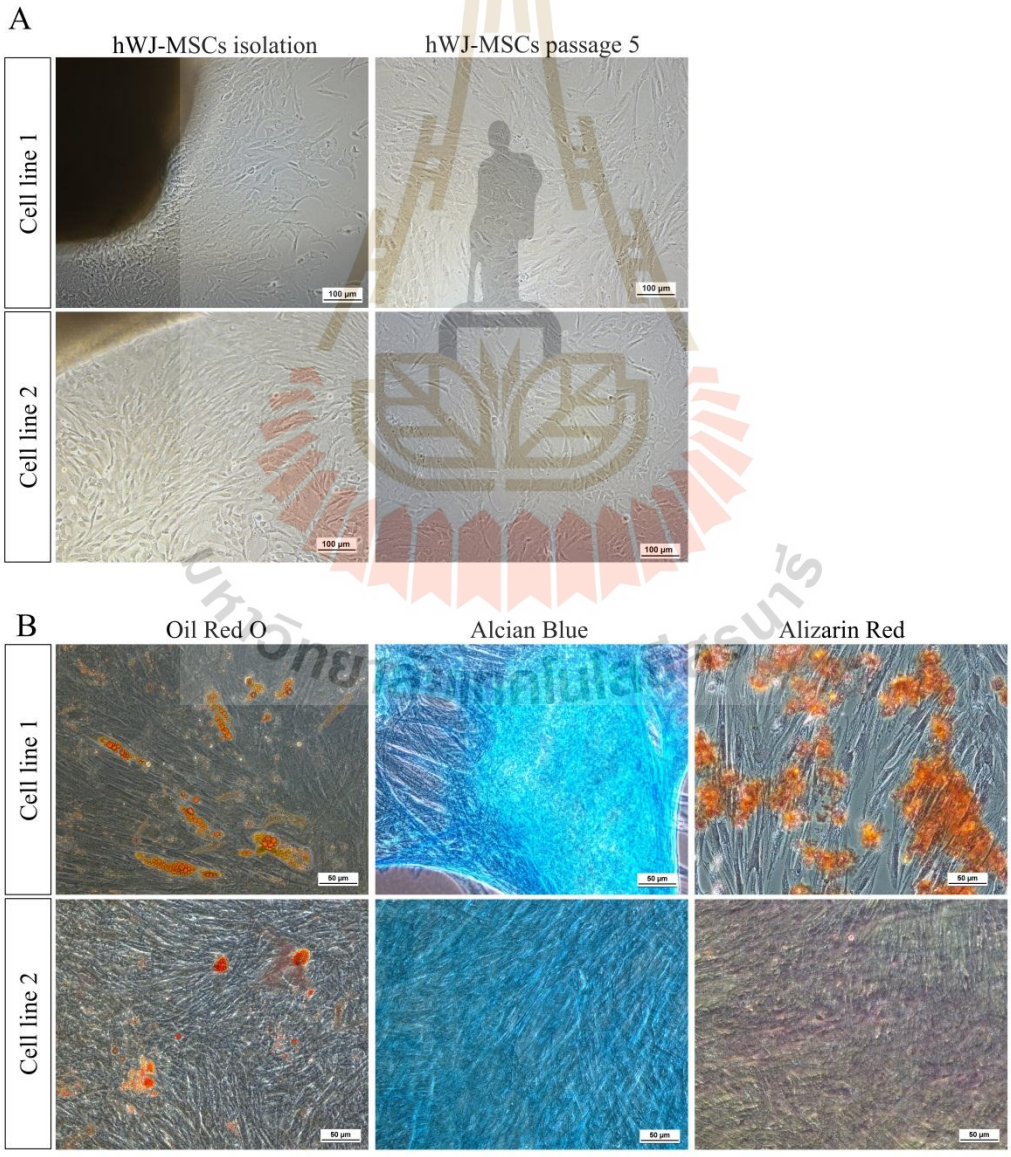

Figure 3.2 MSCs with fibroblast-like morphology. (**A**) Phase contrast images of MSCs expanded from Wharton's Jelly tissue (left) and MSCs were 80% confluences (right) of cell line 1 and 2, scale bar = 100 µm. (**B**) Trilineage differentiation ability of MSCs after 21 days, evaluated by Alizarin Red (osteogenic), Alcian Blue (chondrogenic) and Oil Red O (adipogenic) staining, scale bar = 50 µm.

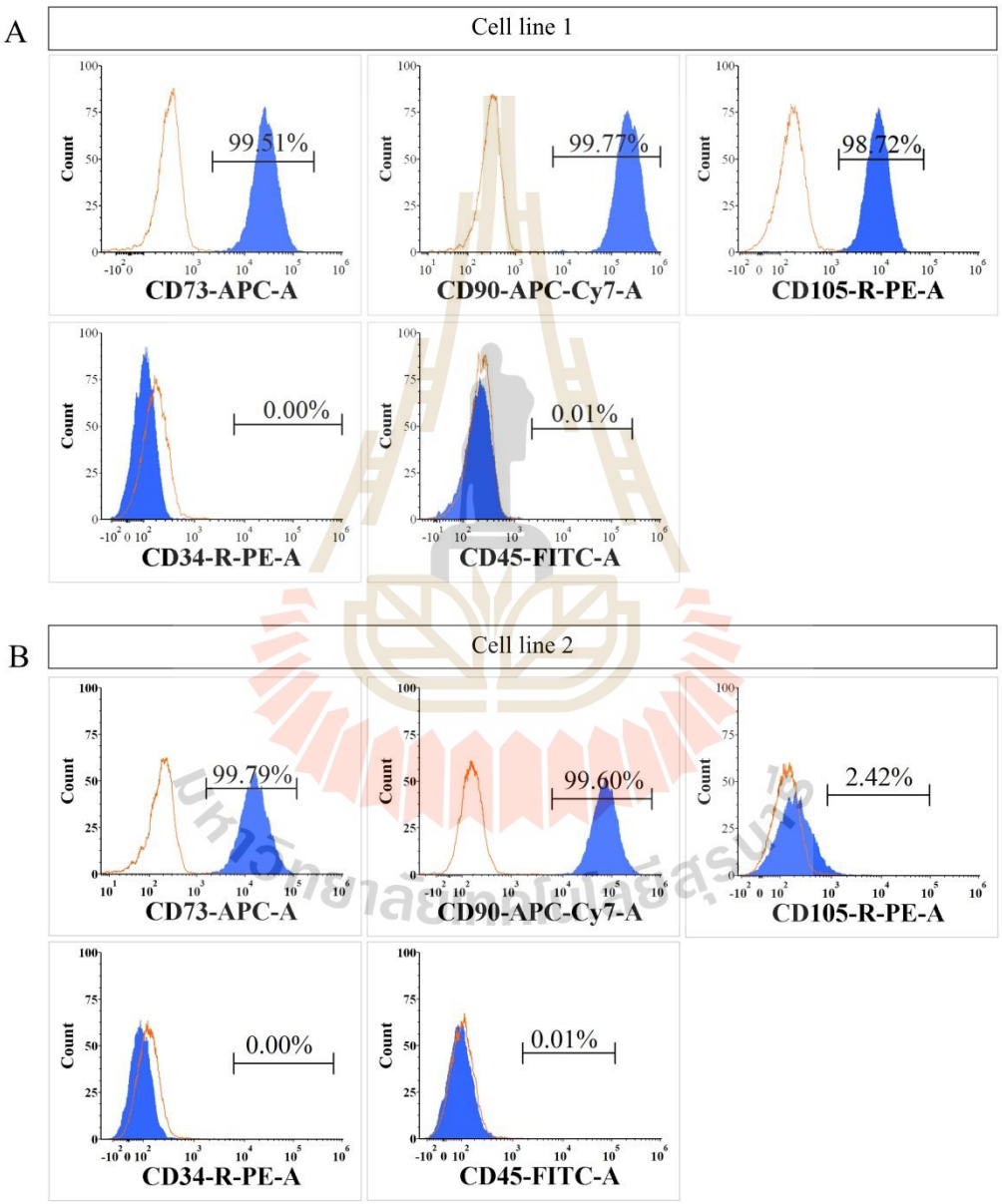

Figure 3.3 Cell surface marker expression analysis of MSCs. MSCs of cell line 1 (**A**) and 2 (**B**) were analyzed by flow cytometry with $CD73^{+}$, $CD90^{+}$, $CD105^{+}$, $CD34^{-}$ and $CD45^{-}$ cell surface marker.

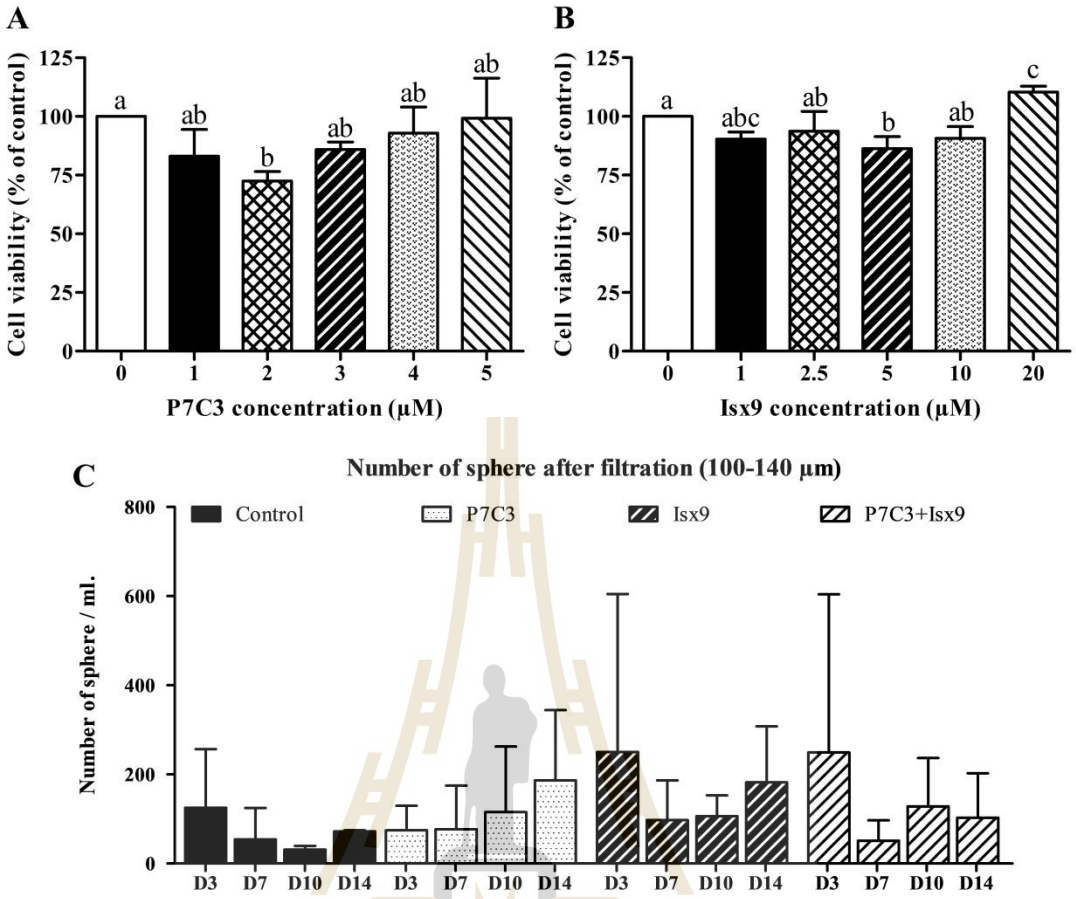

Figure 3.4 Cytotoxicity of MSCs after P7C3 or Isx9 treatment. (**A**) Cell viability was assessed by MTT assay at 0, 1, 2.5, 5, 10, and 20 MM P7C3 treatment for 48h. (B) Cell viability was assessed by MTT assay at 0, 0.25, 0.50, 1, 2.5, and 5 μM Isx9 treatment for 48h. (**C**) Number of spheres after filtration (diameter 100-140 μm). Data were shown as mean±S.D. with different lowercase letters, are significantly different at $p<0.05$.

3.4.2 P7C3 and Isx9 cytotoxicity

Cytotoxicity of small molecules, P7C3 and Isx9 was evaluated by MTT assay (Fig. 3.4A-B). The results of cell viability were compared with not adding any small molecules. The results found highest toxic effect on MSCs at 2 μM P7C3 and 5 uM Isx9 and showed significantly difference compared with control ($p<0.05$). However, the concentrations of 3 μM P7C3 and 10 μM Isx9 were not toxic on MSCs as were used for

neuron induction (Yang et al., 2019). Therefore, we selected P7C3 at 3 μM and Isx9 at 10 μM for further induction of MSCs into the NSs.

3.4.3 Neurosphere characterizations

NSs at approximately diameter of 100-140 um, were counted and recorded at each duration of the experimental groups (Fig. 3.4C). In control and Isx9 group showed high number of spheres on day 3. In the P7C3 group, sphere number was gradually increased from day 3 to day 14. However, in the P7C3+Isx9 group, the numbers of spheres fluctuated up and down in all duration but no significant differences were found when compared in each group.

1) Immunocytochemical staining

NSs were examined with intracellular antigen-specific neuron staining, nestin, SOX2, β-tubulin 3, β-catenin, and DCX, compared with MSCs (Fig. 3.5-3.9). Staining and intensity of immunofluorescence expression results displayed MSCs were differentiated to be NSs. The results showed nestin protein expression progressively high in all group (Fig. 3.10A). Especially, Isx9 group on days 14 was significantly higher than days 3 of other groups (p<0.05). Results of B-tubulin 3 antibody staining (Fig. 3.10B), at day 14 of Isx9 groups was significantly higher than days 3 of P7C3 group (p<0.05). β-catenin staining (Fig. 3.10C), showed high expression in all group, with no significant difference was found in all experimental groups. Similarly, DCX staining results (Fig. 3.10D) for immature neurons, no significant difference was found in all Y experimental groups. Finally, for S0X2 protein (Fig. 3.10E), P7C3 groups exhibited high expression on days 14 which was significantly higher than day 3 in the same group (p<0.05).

2) Gene Expression Analysis

Gene expression analysis of NSs by qPCR, *β-catenin*, *NEUROD1*, *SOX2*, *nestin*, *β-tubulin 3*, and *DCX* genes, were compared to each group and SHSY-5Y neuroblastoma (positive control for neuron cells).

The expression of *β-catenin* genes was showed in Fig. 3.11A. *β-catenin* genes expression of P7C3 group at days 7 and 14 were significantly lower than days 3 of Isx9 group (p<0.05) but no significant difference with the other groups.

NEUROD1 gene expression of P7C3 group at days 3 and 7 and SHSY-5Y were significantly lower than days 10 of Isx9 group (p<0.05) but no significant difference with the other groups.

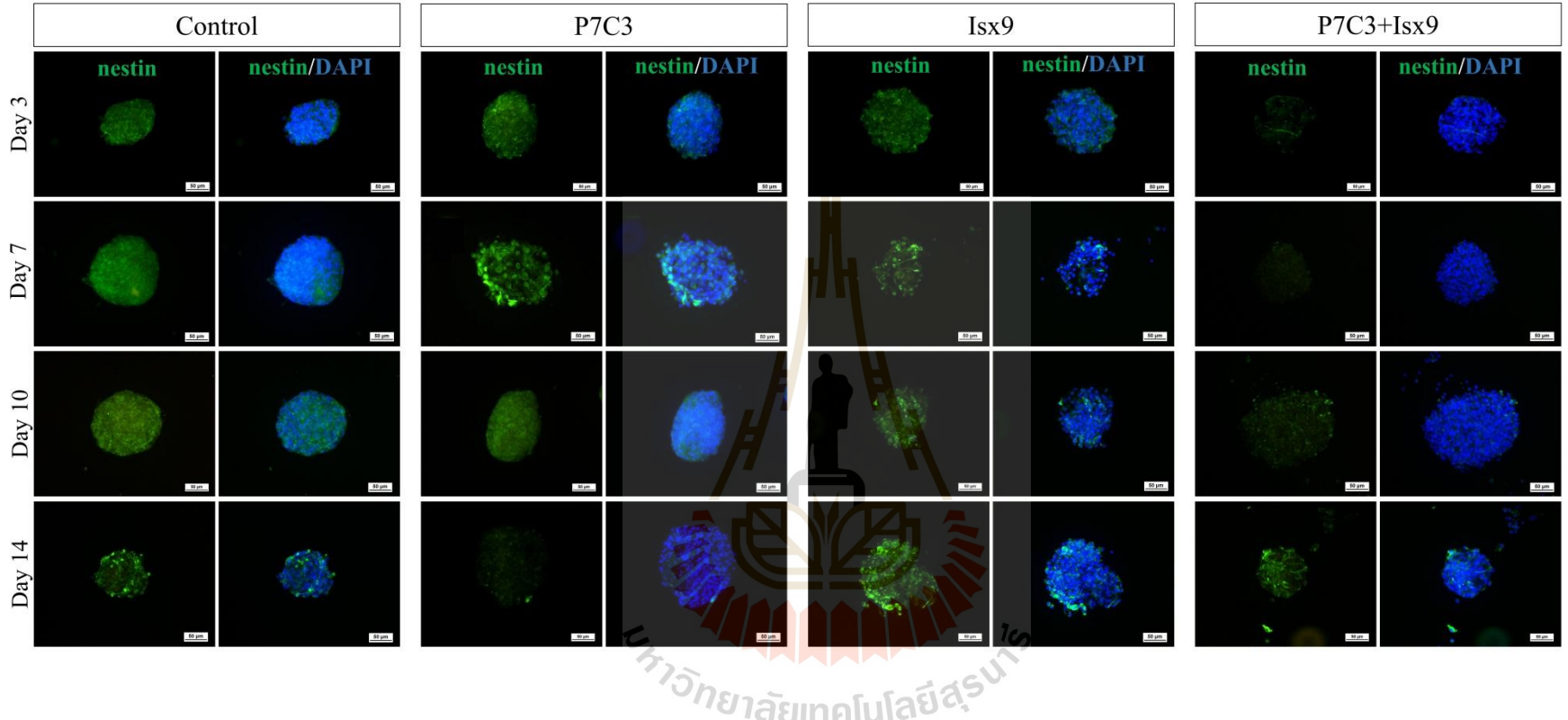

Figure 3.5 ICC staining image of NSs with nestin. Nestin (green; antigen-specific for neural stem/progenitor cell) and DAPI (blue; nucleus) were co-stained to NSs, scale bar = 50 μm.

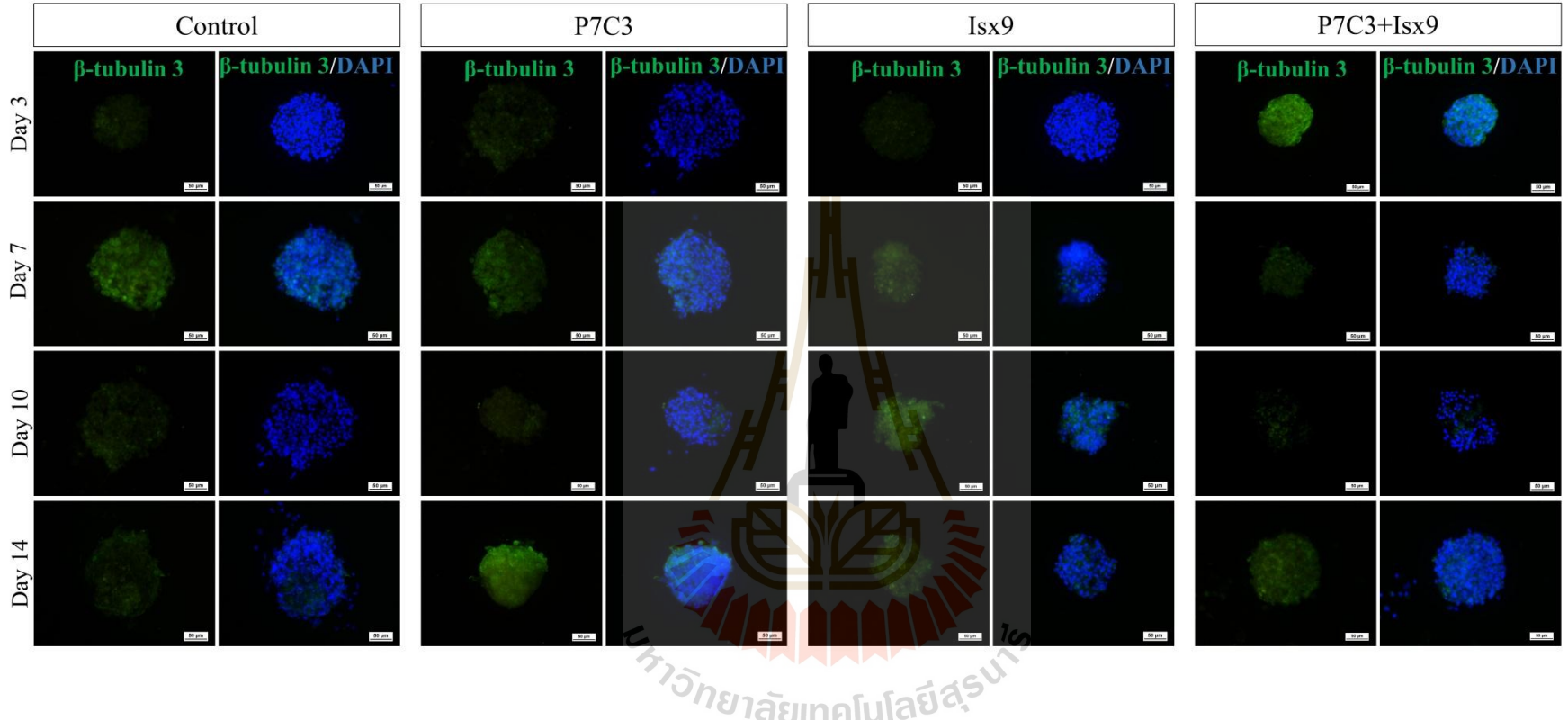

Figure 3.6 ICC staining image of NSs with β-tubulin 3. β-tubulin 3 (green; antigen-specific for neuron cell) and DAPI (blue; nucleus) were co-stained to NSs, scale bar = 50 μm.

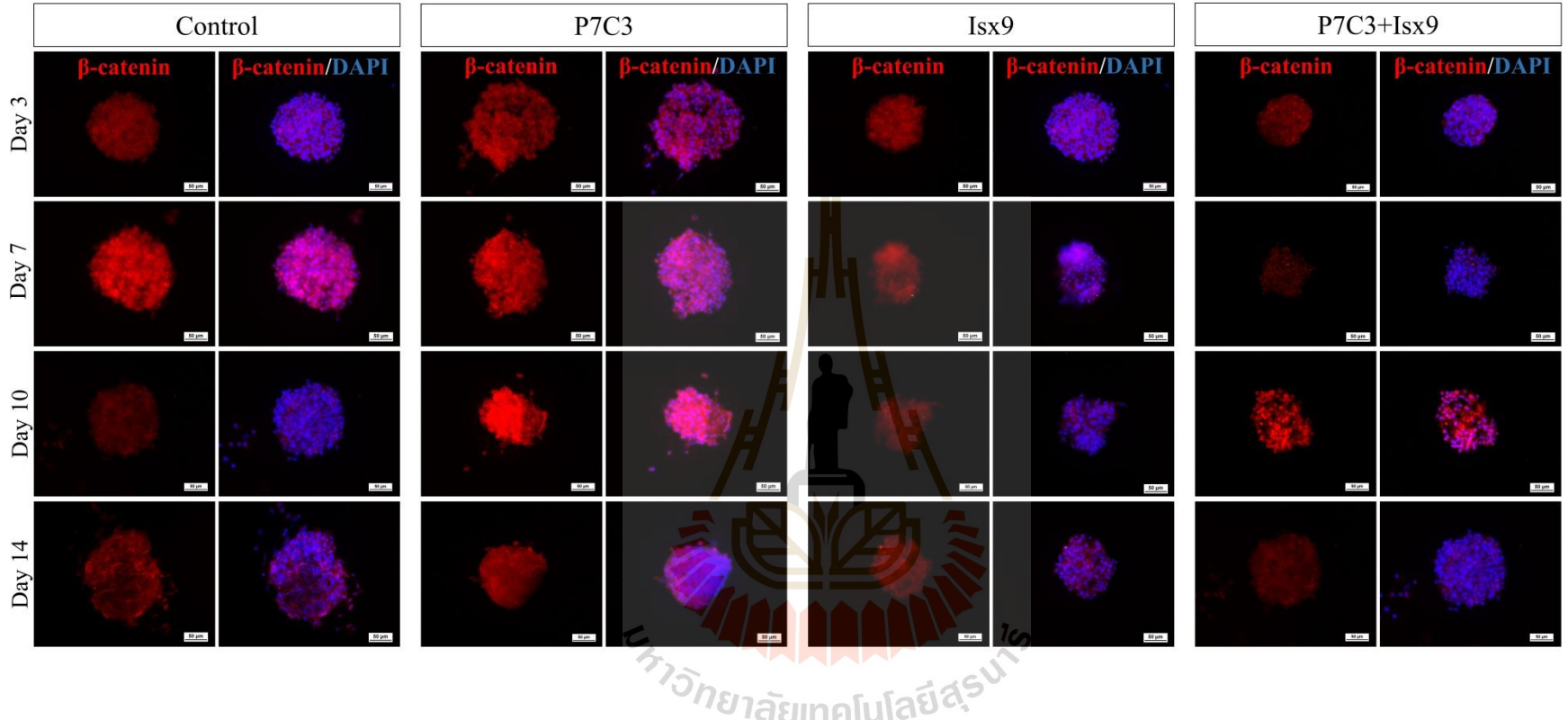

Figure 3.7 ICC staining image of NSs with β-catenin. β-catenin (red; antigen-specific for Wnt3A signaling pathway activation) and DAPI (blue; nucleus) were co-stained to NSs, scale bar = 50 μm.

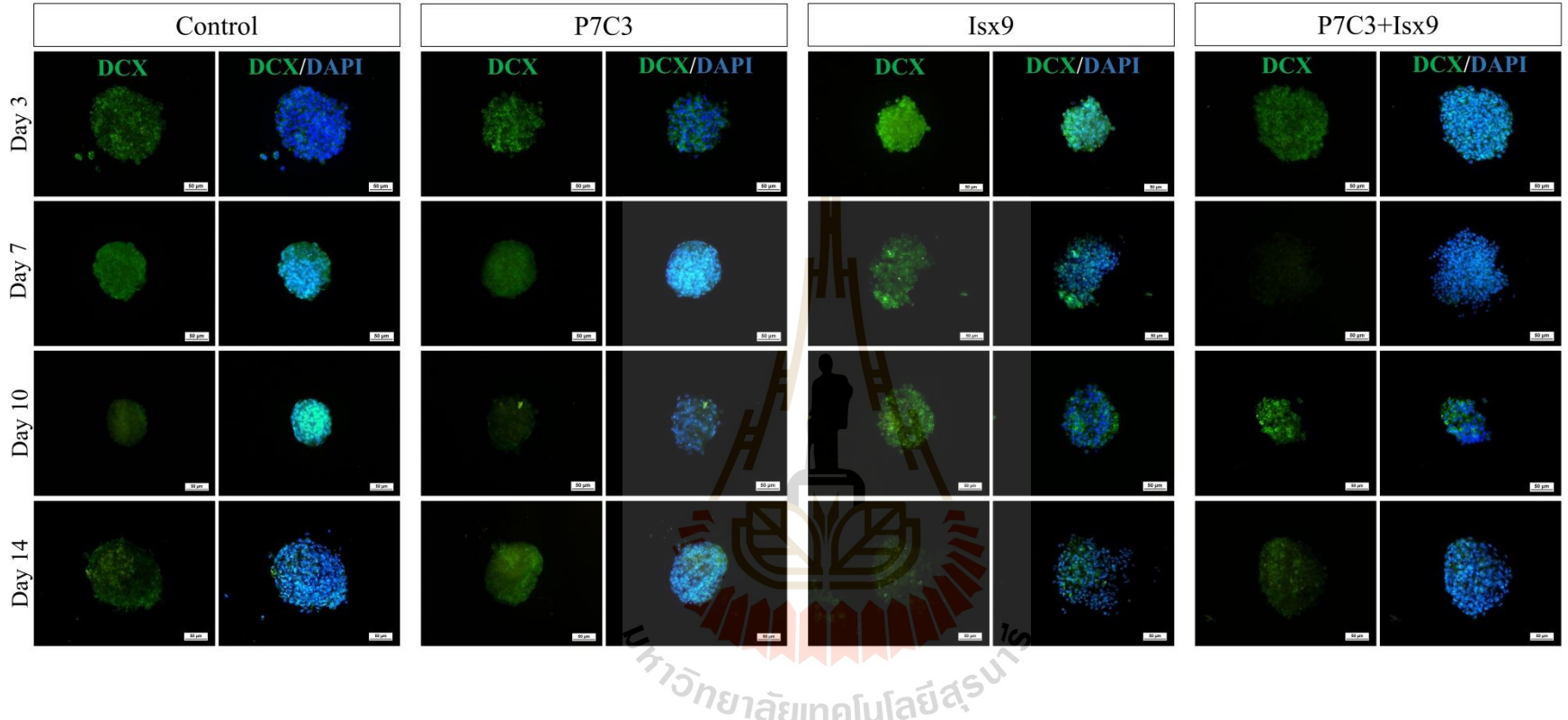

Figure 3.8 ICC staining image of NSs with DCX. DC (green; antigen-specific for immature neuron cell) and DAPI (blue; nucleus) were co-stained to NSs, scale bar = 50 μm.

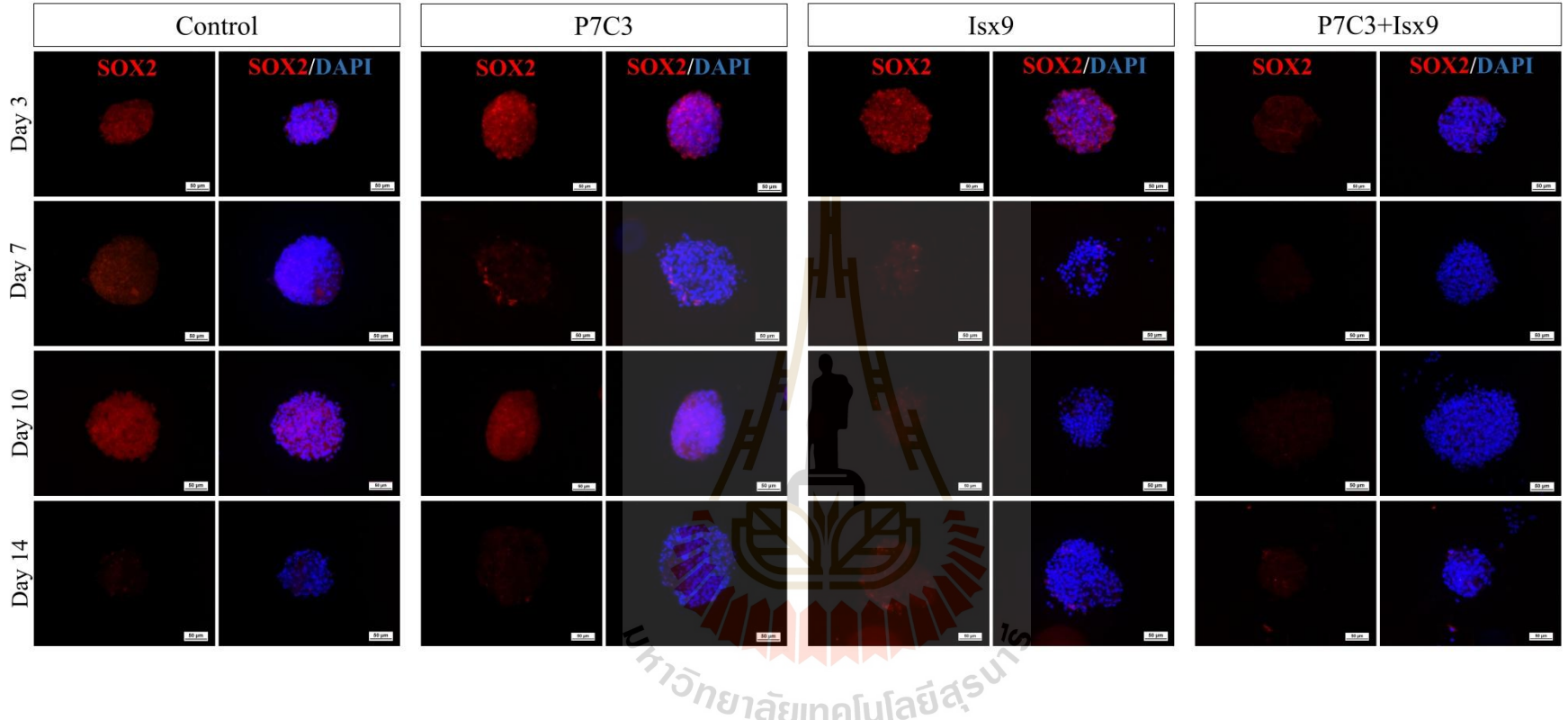

Figure 3.9 ICC staining image of NSs with SOX2. SOX2 (red; antigen-specific for neural stem cell differentiation inhibitor) and DAPI (blue; nucleus) were co-stained to NSs, scale bar = 50 μm.

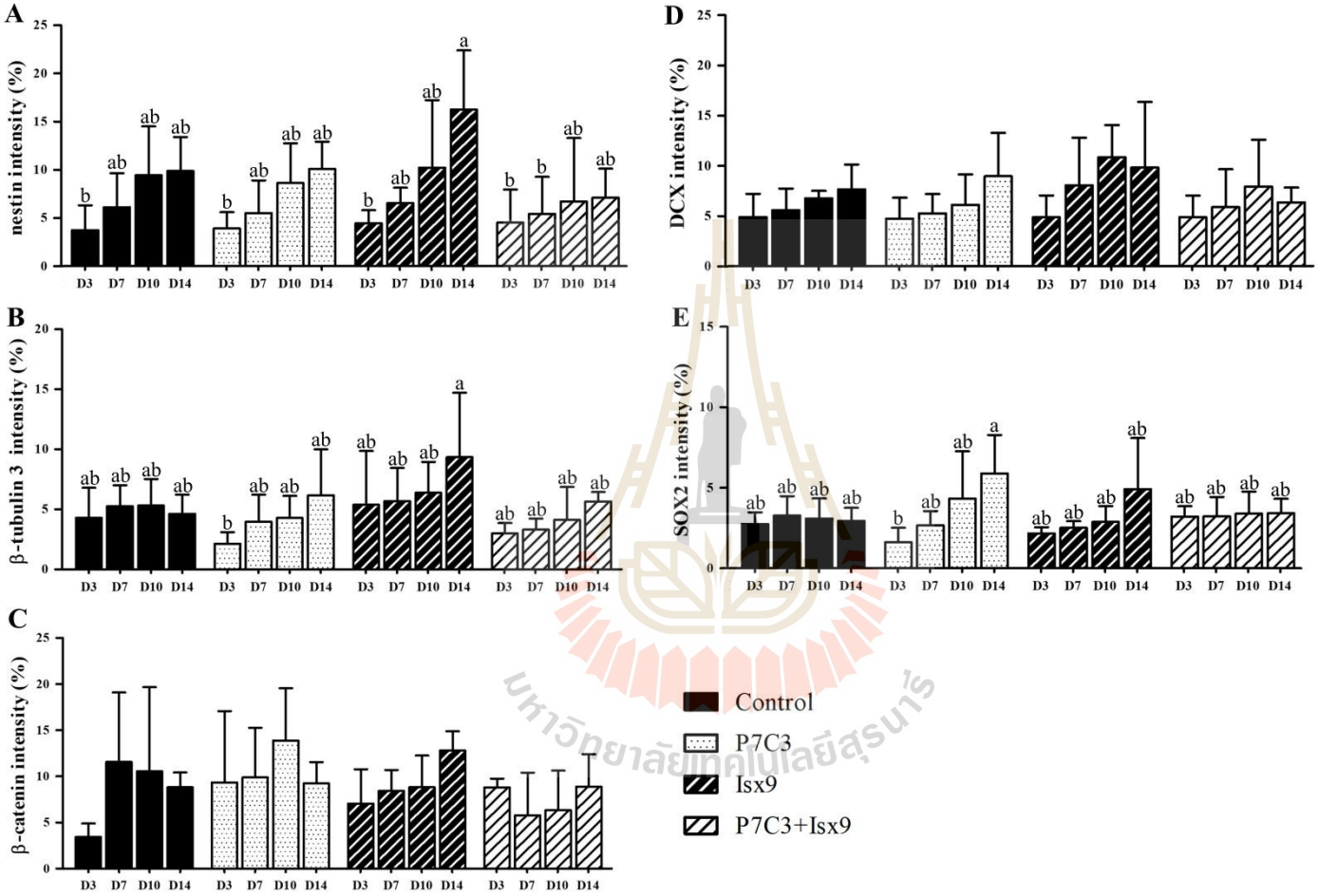

Figure 3.10 Immunofluorescence expression intensity results of NSs. (**A**) Nestin, (**B**) β-tubulin 3, (**C**) β-catenin, (**D**) DCX and (**E**) SOX2 intensity analysis were shown as mean ± S.D. with different lowercase letters, are significantly different at $p<0.05$.

SOX2 gene expression for neural stem cell differentiation inhibitor (Fig. 3.11C) at days 14 of control and P7C3 group were significantly higher than the other groups (p<0.05) but no significant difference with control group at days 7, Isx9 group at day 10 and 14, P7C3+Isx9 group at day 3 and SHSY-5Y group.

The expression of *nestin* genes for neural stem/progenitor cells was showed in Fig. 3.12A. *Nestin* genes expression of P7C3+Isx9 group at days 14 was higher than the other groups (p<0.05) but no significant difference with Isx9 group at day 10 and 14 and P7C3+Isx9 group at day 10.

The expression of *β-tubulin 3* genes for neuron cells was showed in Fig. 3.12B. *β-tubulin 3* genes expression at days 14 of control and P7C3 groups were significantly lower than P7C3 group and P7C3+Isx9 group at day 3, SHSY-5Y and all days of Isx9 group (p<0.05) but no significant difference with the other groups.

The last gene expression results, *DCX* genes expression (Fig. 3.120) were significantly lower than SHSY-5Y (p<0.05) but no significant difference with the other groups.

Gene expression analysis of NSs by qPCR, only Isx9 group at days 7 showed all the expression of *β-catenin*, *nestin*, *NEUROD1* and *β-tubulin 3* genes, higher than the other group. Moreover, Isx9 group at days 7 also low expressed *SOX2* for neural stem cell differentiation inhibitor. Therefore, we selected the NSs from day 7 of Isx9 group for further neurosphere transplantation in rats.

3) Western blotting analysis

Western blots were performed with NSs on day 7 to characterize protein expression followed gene expression results. Nestin, β-catenin, β-tubulin 3, DCX, NEUROD1 and SOX2 protein expression for Wnt3A signaling pathway markers and neural stem/progenitor cell markers were analyzed and compared with control and SHSY-5Y (Fig. 3.13). The intensity changes of β-tubulin 3/ β-actin protein (Fig. 3.14B) in P7C3 and Isx9 groups was significantly lower than SHSY-5Y (p<0.05). However, there were no significen difference in/other groups and the other proteins.

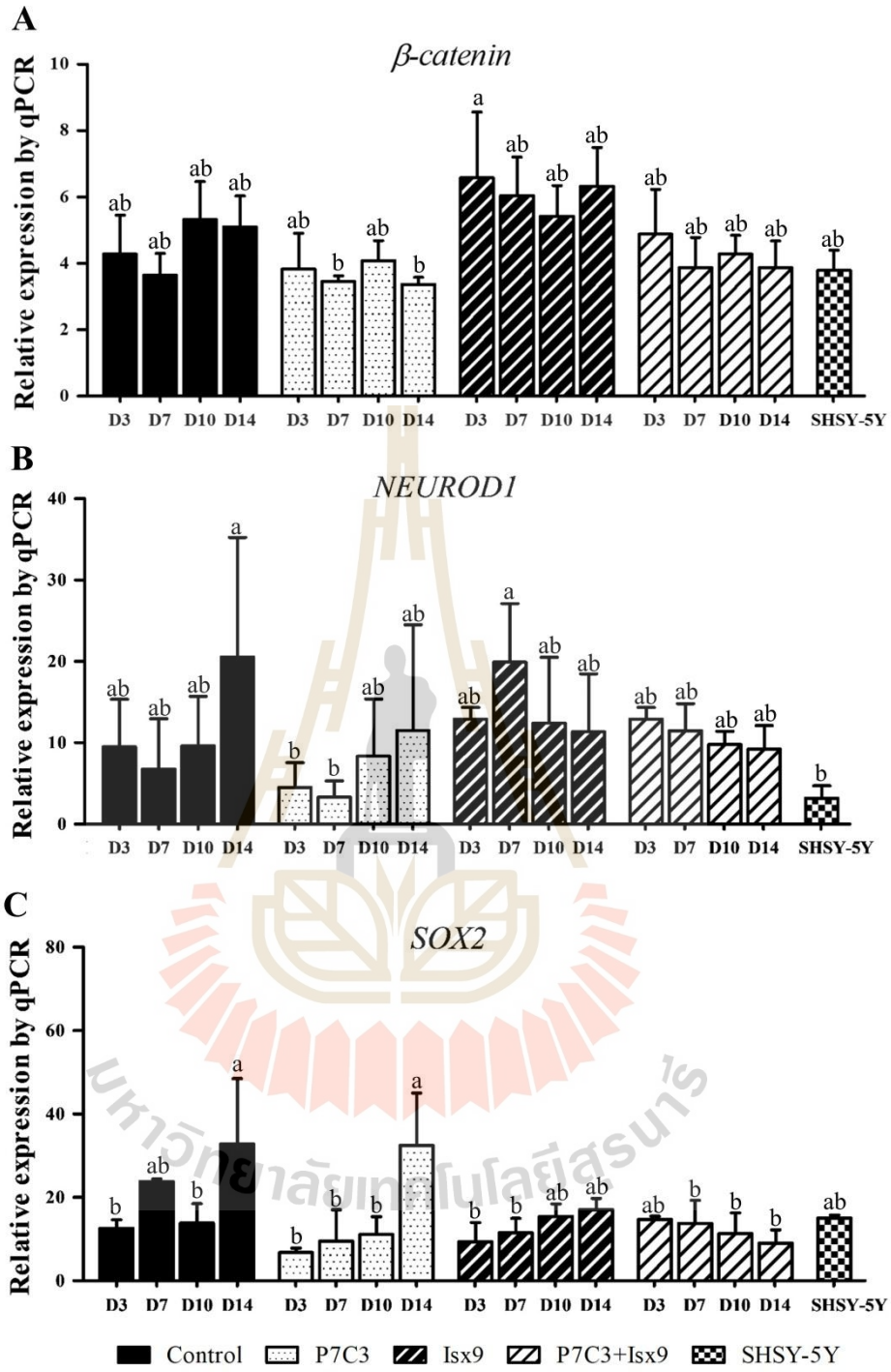

Figure 3.11 Gene expression analysis of NSs by qPCR, (**A**) *β-catenin*, (B) *NEUROD1*, (C) *SOX2* genes. The target gene was normalized to *β-actin* as a reference gene and calculated the expression fold change relative to MSCs. Data were shown as mean±S.D. with different lowercase letters, are significantly different at $p<0.05$.

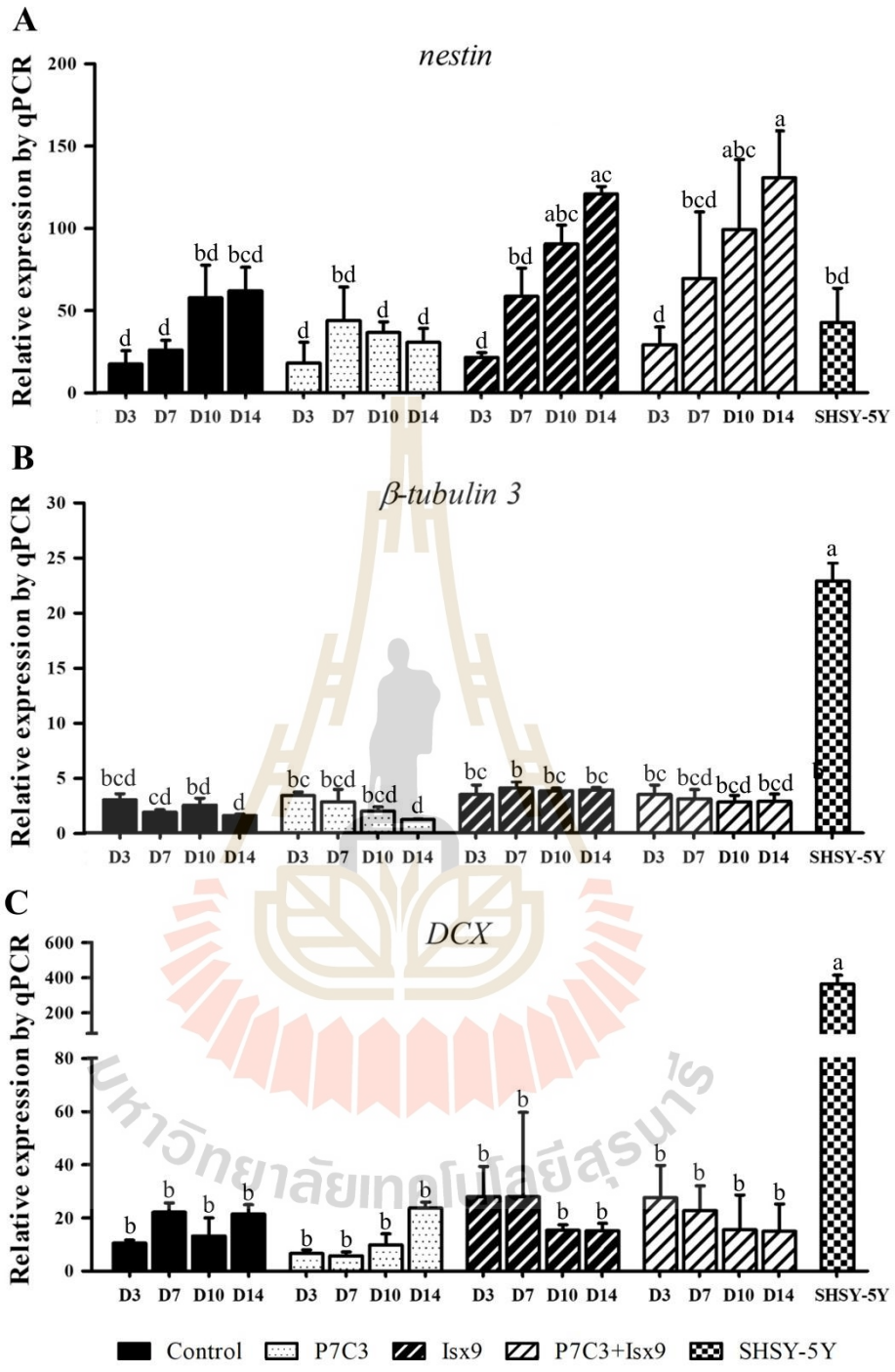

Figure 3.12 Gene expression analysis of NSs by qPCR, (A) *nestin*, (B) *β-tubulin 3*, (C) *DCX* genes. The target gene was normalized to *β-actin* as a reference gene and calculated the expression fold change relative to MSCs. Data were shown as mean±S.D. with different lowercase letters, are significantly different at $p<0.05$.

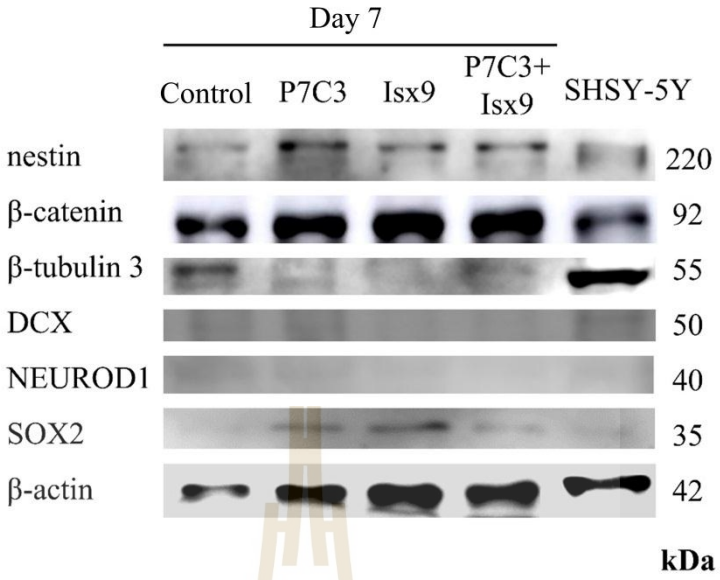

Figure 3.13 Western blot images of nestin, β-catenin, β-tubulin 3, DCX, NEUROD1 and SOX2 protein and β-actin protein.

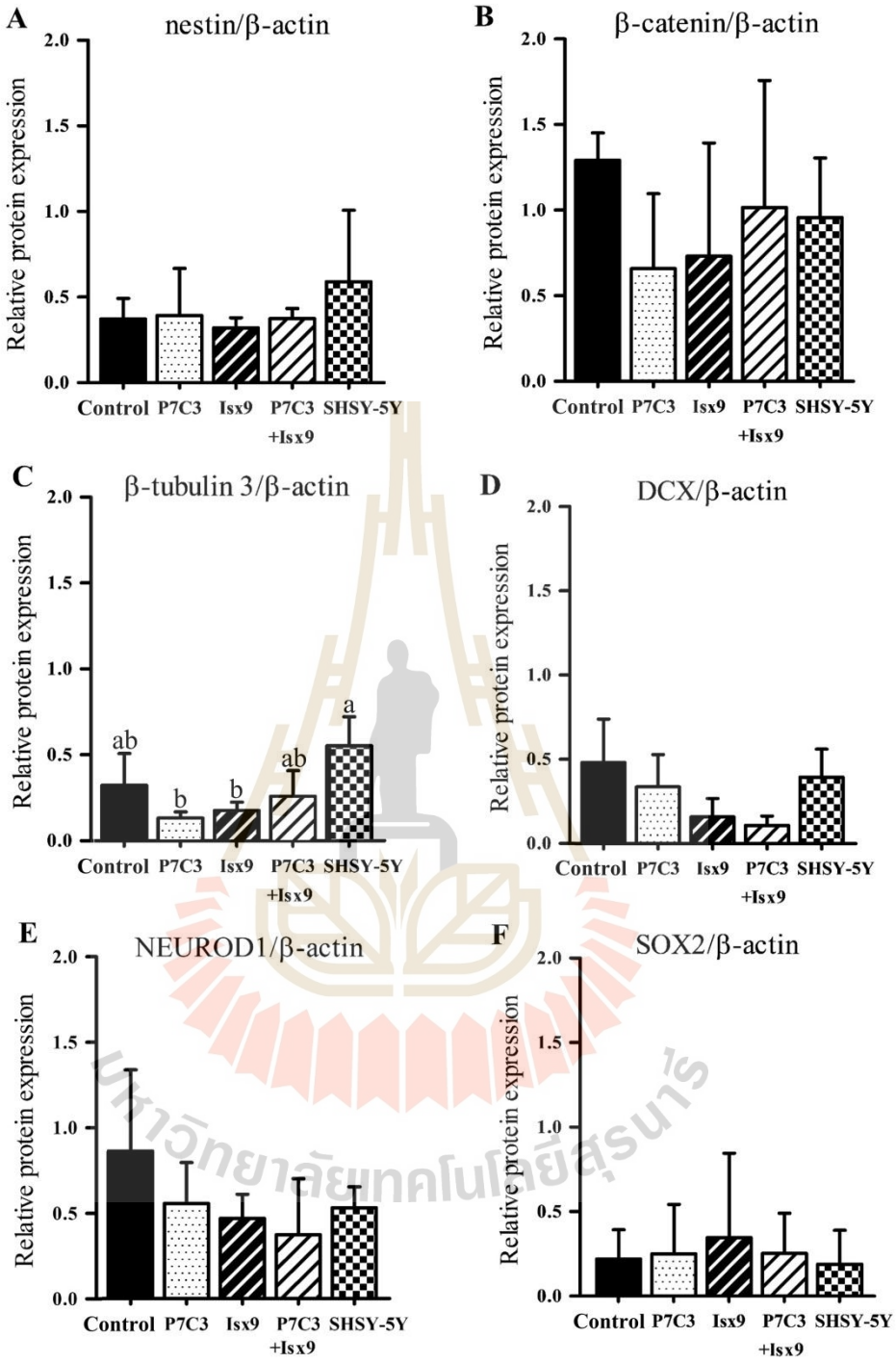

Figure 3.14 Western blot result quantifications of (**A**) nestin, (**B**) β-catenin, (**C**) β-tubulin 3, (**D**) DCX, (**E**) NEUROD1 and (**F**) SOX2 protein and β-actin protein was used as an internal control. Data were shown as mean±S.D. with different lowercase letters, are significantly different at $p<0.05$.

3.5 Discussion

In this study Wu MSCs were isolated and expanded rom 2 cell lines. Only cell line 1 was accepted for MSCs characterization to use in further experiments. Then, we demonstrated that MSCs were able to differentiate into NSs by using neurogenesis-enhancing small molecules, P7C3-A20 and Isx9. After differentiation, NSs on day 3 of control group and Isx9 group showed the highest number of spheres. This result of NSs represented cell proliferation with/without Isx9. Similarly, numbers of small NSs had formed within 3-5 days from IDPSCs differentiation (Kawase Koga et al, 2020). The average diameter of primary NSs was 97 μm with a capacity for self-renewal. However, only the high number of spheres could not show the NSs characterization, therefore gene and protein expression were performed to identify NSs characterization in this study.

NSs were characterized by ICC. The result showed gradually increase nestin protein expression at day 3 and highest at days 14 in the Isx9 groups. Nestin protein expression represented MSCs could differentiate to neural stem/progenitor cells (Hosseini et al., 2018). Similar with the result of NSs were differentiated from hDPSCs for 14 days also found high expression of nestin (Kawase Koga et al., 2020). For SOX2 protein results showed highest expression on days 14 of P7C3 group. However, those SOX2 protein in this study, was inhibited NSCs proliferation via Wnt3A signaling pathway from many signaling in the induction media such as bFGF, P7C3, and Isx9 to activated NSCs differentiation (Shohayeb et al., 2018; Vanderhaeghen, 2009). Although, the results of β-catenin and DCX staining showed theexpression in all experimental groups, but no statistically different in all experimental groups. For β-catenin, because of β-catenin protein is activated by a lot of signaling through many proliferation and differentiation pathways. Moreover, Wnt3A signaling pathway followed by NSCs differentiation and nestin protein expression, need specific signal and time to going toward terminal differentiation or maturation (Hagihara et al., 2019; Shohayeb et al., 2018). Likewise, the results of β-tubulin 3 protein expression, neuron cells, also showed gradually increase and highest expression on days 14 of Isx. Group but no significant different when compared to other duration of induction. β-tubulin 3 protein expression on day 14 also displayed terminal differentiation in this study.

Protein expression on day 7 after induction was analyzed by western blots analysis. The results of P7C3 and Isx9 groups found that relative protein expression of β-tubulin 3/ β-actin protein was significantly lower than SHSY-5Y. On the contrary with previous study, P7C3 and Isx9 small molecule showed high - tubulin 3 protein expression by ICC result of neuron differentiation from human skin fibroblasts (Yang et al., 2019). Unlike our results that no protein expression like mature neuron.

Furthermore, the NSs showed all gene expression of *β-catenin*, *nestin*, *NEUROD1* and *β-tubulin 3* genes in Isx9 group at 7 days higher than other groups. Similarly, the previous results showed the high expression of *nestin* as multiple neurogenic genes after induction. Besides, they reported that the transcriptional pattern of NSs was differentiated from both MSCs and hNSCs (Peng et al., 2019). Likewise, previously reported the induction of MSCs to NSs showed high *β-tubulin 3* gene expression start on day 4 of induction. Gene expression of this study could predict to transcriptional regulation of adult neurogenesis (Bonilla-Porras et al, 2017) Isx9 group at 7 days also expressed *NEUROD1* gene which is an important transcription factor involved for neuronal development and maturation. The production of *NEUROD1* gene such as nestin and β-tubulin 3 protein also found in NSs from Isx9 group at 7 days. For neurogenesis, Wnt3A signaling (Fig. 3.15) prevent the SOX2-dependent regulation of *NEUROD1* gene transcription (Arredondo et al, 2020; Shohayeb et al., 2018). Therefore, low expression of SOX2 marker is important point of NSs induction by Isx9 at 7 days. Thus, we selected the NSs from day 7 of Isx9 group for further NS cell transplantation in rats. Because of the neural stem/progenitor cells of this group that we found on gene and protein expression results.

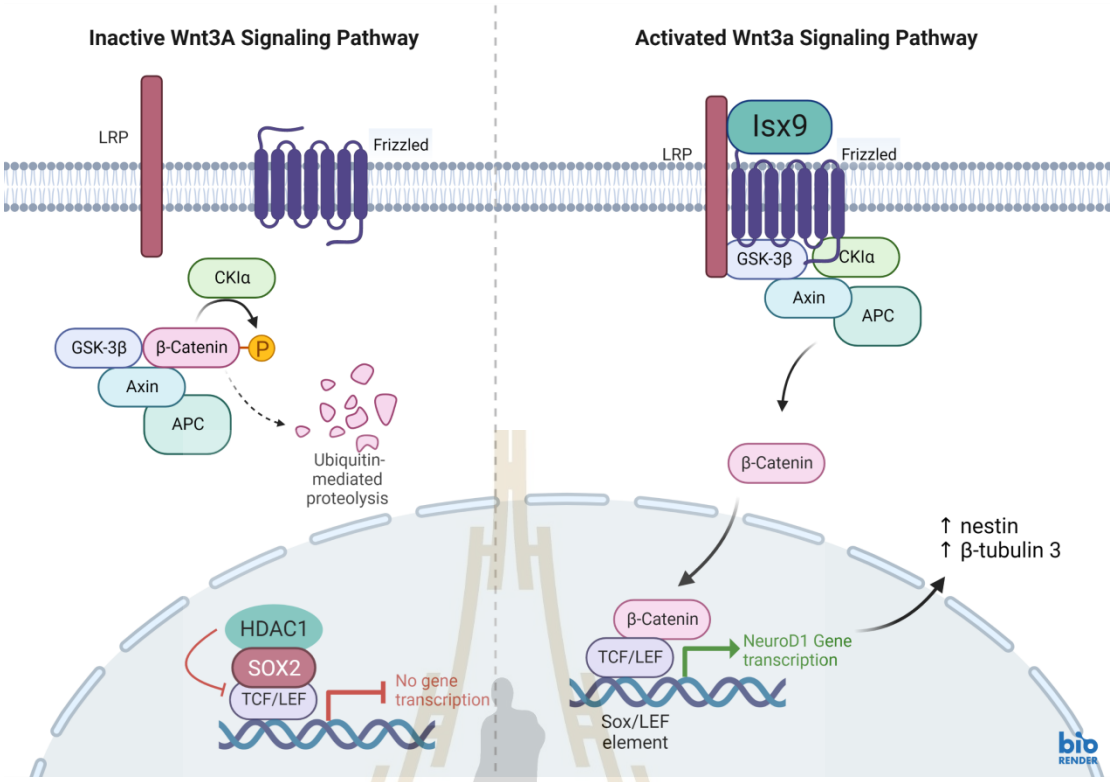

Figure 3.15 Wnt3A signaling pathway. (left) Inactive Wnt3A signaling pathway, β-catenin are degraded by multiprotein complex of ubiquitin-mediated proteolysis. (right) Activated Wnt3A signaling pathway, when the small molecule isx9 binds to the LRP (lipoprotein receptor), then a multiprotein complex composed of Axin, APC, CK1, and GSK-3B is activated and β-catenin is released. β-catenin translocate through the nucleus and interacts with TCF/LEF transcription factor. While the repressor protein complex, HDAC1 and SOX2, is degraded. β-catenin and TCF/LEF regulate the transcription of target genes. *NEUROD1* gene transcription (neurogenic differentiation 1; NEUROD1), the target gene, activated neurogenic differentiation marker such as nestin and β-tubulin3. Created with BioRender.com.

3.6 Conclusions

The differentiation of hWJ-MSCs into NSs by neurogenesis enhancing P7C3-A20 and Isx9 was studied. Induction media composed of 10 μM Isx9 could induce hWJ-MSCs to be NSs through Wnt3A signaling pathway. β-catenin marker was activated by Isx9 through the Wnt3A signaling pathway. In the same way, β-tubulin 3 and nestin markers

showed gradually increased expression, indicating MSCs could differentiate into neuron cells and neural stem/progenitor cells. These NSs showed both neural stem/progenitor cells by gene and protein expression. Seven days after induction in Isx9 media NSs were selected to be used in the treatment of spinal cord injury in rat model.

3.7 References

Arredondo, S. B., Valenzuela-Bezanilla, D., Mardones, M. D., & Varela-Nallar, L. (2020). Role of Wnt signaling in adult hippocampal neurogenesis in health and disease. **Frontiers in Cell and Developmental biology**, *8*. doi:10.3389/fcell.2020.00860.

Balasubramanian, S., Thej, C., Venugopal, P., Priya, N., Zakaria, Z., Sundarraj, S., & Majumdar, A. S. (2013). Higher propensity of Wharton's jelly derived mesenchymal stromal cells towards neuronal lineage in comparison to those derived from adipose and bone marrow. **Cell Biology International**, *37*(5), 507-515. doi:10.1002/cbin.10056.

Bonilla-Porras, A. R., Velez-Pardo, C., & Jimenez-Del-Rio, M. (2017). Fast transdifferentiation of human Wharton's jelly mesenchymal stem cells into neurospheres and nerve-like cells. **Journal of Neuroscience Methods**, *282*, 52-60. doi:10.1016/j.jneumeth.2017.03.005.

George, S., Hamblin, M. R., & Abrahamse, H. (2019). Differentiation of Mesenchymal Stem Cells to Neuroglia: in the Context of Cell Signalling. **Stem Cell Reviews and Reports**, *15*(6), 814-826. doi:10.1007/s12015-019-09917-z.

Hagihara, H., Murano, T., Ohira, K., Miwa, M., Nakamura, K., & Miyakawa, T. (2019). Expression of progenitor cell/immature neuron markers does not present definitive evidence for adult neurogenesis. **Molecular brain**, *12*(1), 108. doi:10.1186/s13041-019-0522-8.

Hosseini, S. M., Sani, M., Haider, K. H., Dorvash, M., Ziaee, S. M., Karimi, A., & Namavar, M. R. (2018). Concomitant use of mesenchymal stem cells and neural stem cells for treatment of spinal cord injury: A combo cell therapy approach. **Neuroscience Letters**, *668*, 138-146. doi:10.1016/j.neulet.2018.01.008.

Kawase-Koga, Y., Fujii, Y., Yamakawa, D., Sato, M., & Chikazu, D. (2020). Identification of neurospheres generated from human dental pulp stem cells in xeno-/serum-free conditions. **Regenerative therapy**, *14*, 128-135. doi:10.1016/j.reth. 2019. 11.006.

Kim, D. W., Staples, M., Shinozuka, K., Pantcheva, P., Kang, S. D., & Borlongan, C. V. (2013). Wharton's jelly-derived mesenchymal stem cells: phenotypic characterization and optimizing their therapeutic potential for clinical applications. **International Journal of Molecular Sciences**, *14*(6), 11692-11712. doi:10.3390/ijms140611692.

Malgieri, A., Kantzari, E., Patrizi, M. P., & Gambardella, S. (2010). Bone marrow and umbilical cord blood human mesenchymal stem cells: state of the art. **International Journal of Clinical and Experimental Medicine**, *3*(4), 248-269.

Nori, S., Okada, Y., Nishimura, S., Sasaki, T., Itakura, G., Kobayashi, Y., . . . Okano, H. (2015). Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: oncogenic transformation with epithelial-mesenchymal transition. **Stem Cell Reports**, *4*(3), 360-373. doi:10.1016/j.stemcr.2015.01.006.

Panta, W., Imsoonthornruksa, S., Yoisungnern, T., Suksaweang, S., Ketudat-Cairns, M., & Parnpai, R. (2019). Enhanced hepatogenic differentiation of human Wharton's jelly-derived mesenchymal stem cells by using three-step protocol. **International Journal of Molecular Sciences**, *20*(12). doi:10.3390/ ijms20123016.

Peng, C., Li, Y., Lu, L., Zhu, J., Li, H., & Hu, J. (2019). Efficient one-step induction of human umbilical cord-derived mesenchymal stem cells (UC-MSCs) produces MSC-derived neurospheres (MSC-NS) with unique transcriptional profile and enhanced neurogenic and angiogenic secretomes. **Stem Cells International**, 2019, 9208173. doi:10.1155/2019/9208173.

Schneider, J. W., Gao, Z., Li, S., Farooqi, M., Tang, T.-S., Bezprozvanny, I., . . . Hsieh, J. (2008). Small-molecule activation of neuronal cell fate. **Nature Chemical Biology**, *4*(7), 408-410. doi:10.1038/nchembio.95.

Shohayeb, B., Diab, M., Ahmed, M., & Ng, D. C. H. (2018). Factors that influence adult neurogenesis as potential therapy. **Translational Neurodegeneration**, *7*(1), 4. doi:10.1186/s40035-018-0109-9.

Sun, L., Wang, F., Chen, H., Liu, D., Qu, T., Li, X., . . . Chen, Y. (2019). Co-transplantation of human umbilical cord mesenchymal stem cells and human neural stem cells improves the outcome in rats with spinal cord injury. **Cell Transplantation**, *28*(7), 893-906. doi:10.1177/0963689719844525.

Vanderhaeghen, P. (2009). Wnts blow on NeuroD1 to promote adult neuron production and diversity. **Nature Neuroscience**, *12*(9), 1079-1081. doi:10.1038/nn0909- 1079.

Venkatesh, K., & Sen, D. (2017). Mesenchymal Stem Cells as a Source of Dopaminergic Neurons: A Potential Cell Based Therapy for Parkinson's Disease. **Stem Cell Research & Therapy**, *12*(4), 326-347. doi:10.2174/ 1574888x 12666 16111 4122059.

Yang, Y., Chen, R., Wu, X., Zhao, Y., Fan, Y., Xiao, Z., . . . Dai, J. (2019). Rapid and efficient conversion of human fibroblasts into functional neurons by small molecules. **Stem Cell Reports**, *13*(5), 862-876.

Yang, Y., Fan, Y., Zhang, H., Zhang, Q., Zhao, Y., Xiao, Z., . . . Dai, J. (2021). Small molecules combined with collagen hydrogel direct neurogenesis and migration of neural stem cells after spinal cord injury. **Biomaterials**, *269*, 120479.doi:10.1016/j.biomaterials.2020.120479.

Zhou, H. L., Zhang, X. J., Zhang, M. Y., Yan, Z. J., Xu, Z. M., & Xu, R. X. (2016). Transplantation of Human Amniotic Mesenchymal Stem Cells Promotes Functional recovery in a rat Model of Traumatic Spinal Cord Injury. **Neurochemical Research**, *41*(10), 2708-2718. doi:10.1007/s11064-016-1987-9.

CHAPTER IV
TRANSPLANTATION OF NEUROSPHERES TO RECOVER SPINAL CORD INJURY IN RAT

4.1 Abstract

Stem cell research is crucial for the therapeutic treatment of neurodegenerative diseases such as SCI. Steroids were widely administered to SC patients to limit neuronal death. Various studies have shown the potential of using stem cells to repair SCI. This study aims to investigate the transplantation of NSs derived from hWJ-MSCs into a rat model of SCI by regulating and improving remyelination. In the transplantation treatment for SCI, NSs were used. Behavioral assessment of rats and examination of histological analyses were studied. After transplantation for 8 weeks, histological studies showed that MSCs and NSs were found in the damaged spinal cord region and also with neurotransmitter activity (acetylcholine neurotransmitter marker). The expression of GFAP (astrocytes) was found around the spinal cord injury areas in normal saline, MSCs and NSs s groups, representing the glia scar surrounding the spinal cord injury area. However, only the transplanted NSs group expressed immunoreactivity and neuroregeneration through BDNF results. Besides, NSs transplanted rats displayed the smallest cavity size of SCI tissue, resulting in regeneration and repair in the tissue. Along with behavioral tests, NSs-transplanted rats from this work could improve the recovery movement in SCI rat model in 8 weeks.

4.2 Introduction

Neurodegenerative diseases or neurological injuries such as SCI, vascular dementia (VaD) and Alzheimer's disease (AD), etc. The pathologies are losing nerve cells and nerve damage in each region of the nervous system. Falls, motor vehicle accidents, sports injuries, work-related injuries, and communal violence are the most

major causes of SCI, followed by inflammation and cell degradation in the injury location (Leung, 2012). SCI also caused several extra severe medical issues, such as respiratory, urogenital, and skin abnormalities (Hagen, 2015). Furthermore, SCI patients are often young and require prolonged medical and social care, leading to significant socioeconomic issues (DeVivo, 1997). Steroids were often administered to SCI patients to reduce existing cell loss. Several research and preclinical studies on the therapy of SCI using stem cells have been explored all around world (Yamazaki, Kawabori, Seki, & Houkin, 2020). NSs, which were transdifferentiated from hWJ-MSCs from this study, were used. The purpose of this research is to investigate hWJ-MSCs derived- NSs transplantation into the SCI rat model by regulating and enhancing neurogeneration.

4.3 Materials and methods

4.3.1 Experimental animals

Adult male Sprague Dawley (SD) rats (weight, 250-300 g or 8-week-old) were used in this study. All rats in this study were obtained from Nomura Siam International Co., Ltd., kept in standard conditions under a 12-h light/dark cycle, and provided food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee at Suranaree University of Technology (8/2018).

Timeline of SCI model and transplantation show in Fig. 4.1. Fifty male SD rats were selected and randomly divided into five groups, 10 rats/group: (1) sham group; the rats were operated but had no SCI induction, (2) control group; the rats were operated to induce SCI and no cell transplants were performed, (3) normal saline group; the rats were operated to induce SCI and vehicle treatment, (4) MSCs group; the rats were operated to induce SCI and received hWJ-MSCs transplantation, (5) NSs group; the rats were operated to induce SCI and received NSs transplantation.

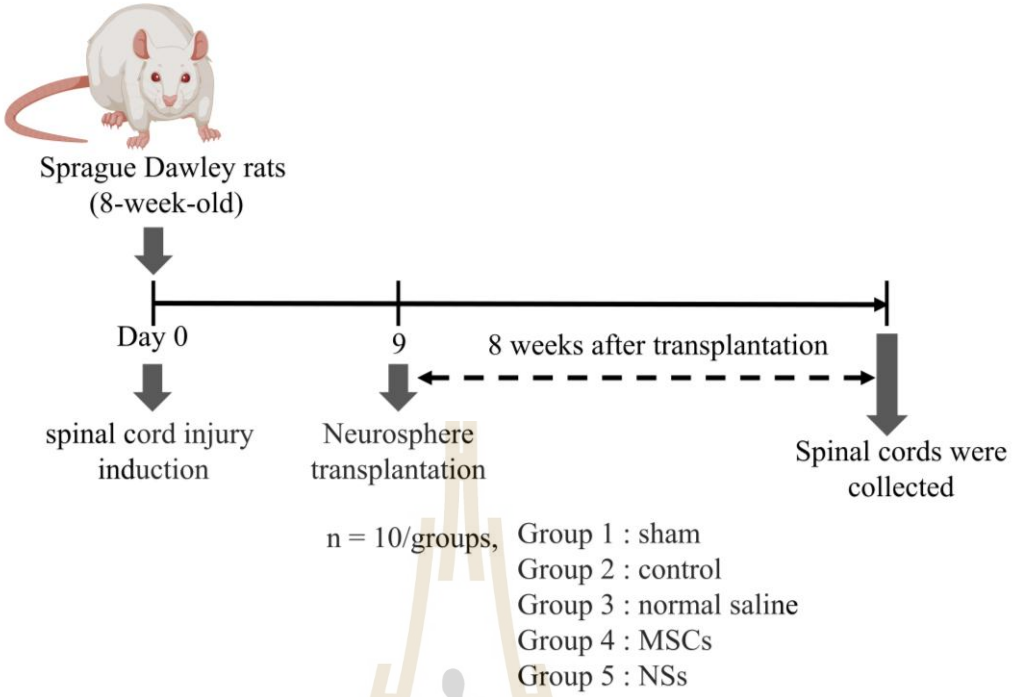

Figure 4.1 Schematic diagram of research. Spinal cord injury transplantation timeline.

4.3.2 Spinal cord injury model

The rat compression SCI model was performed according to procedures previously described (Blight, 1991). Rats were anesthetized using a mixture of 1-3% isoflurane (Aerrane isoflurane, Baxter Healthcare Corporation, Deerfield, Illinois, USA) and 500 ml/min oxygen and maintained throughout surgery. Under sterile technique, compression SCI model was created by forceps compression, calibrated forceps with a spacer (1.3 mm) were used to bilaterally compress to the dura of the spinal cord at thoracic level 9 (T9), for 15 sec (Hosseini et al., 2018; Nori et al., 2018). Rats in sham group were operated with no SCI induction. Post operation, the rats were injected with 5 mg/kg tramadol hydrochloride, 5 mg/kg carprofen, 5 mg/kg enrofloxacin and 3.5 ml 0.9% normal saline, subcutaneous injections. Manual bladder expression was performed 3 times/daily until voluntary urination was established combination with rats were injected with 25 mg/kg sulfamethoxazole trimethoprim, subcutaneous injections.

4.3.3 Cell transplantations

NSs at day 7 from Isx9 group were digested by 0.25% trypsin/EDTA at 37°C, 5 min. Then, NSs and MSCs were harvested, washed by PBS(-), and prepared on ice until transplantation. The total concentration of cells for each group was 1x105 cells in 3 ml normal saline. Cells transplantation was randomly performed at 9 days post-injury. Rats were anesthetized as described above under sterile technique, and the spinal dura matter at T9 were exposed. Stem cells were intramedullary injected into the spinal cord using Hamilton's needles (Hamilton Bonaduz AG, Bonaduz, Switzerland) as described previously (Wu et al., 2011).

For NSs and MSCs group, 3 ul of stem cells (1×10^5 cells) suspension were transplanted into the spinal cord with approximately 1.5 mm deep from the dorsal surface of the dura mater. For normal saline group, rats were injected with 0.9% normal saline in the same protocol. The last group, sham group, the rats were not received any specific treatment, only the spinal dura matter was exposed. All rats received the same medication as before.

4.3.4 Behavioral Tests

BBB behavioral test method was used to evaluate the locomotor recovery of the SCI animals. It was developed by Basso, Beattie, and Bresnahan (1995). This method is a reliable and sensitive locomotor rating scale. BBB behavioral test provides investigators with the performance measurement of behavioral outcomes after SCI and treatment (Basso et al., 1995). Briefly, all rats were placed on a floor in a square enclosure and recorded for 5 min. Their hindlimb locomotor functions were scored using a 22-point (0-21) scale which evaluated parameters including hindlimb joint movement, paw placement, weight support, forelimb hindlimb coordination, etc. Eight rats were selected randomly from each group and were evaluated with BBB behavioral test 1 day before and after the SCI, and every week until the experiment was completed. The scores were obtained by averaging both hindlimb values.

4.3.5 Histology

To evaluate the pathology after 8 weeks of cell transplantation into the injured spinal cords. All rats were euthanized by carbon dioxide fumigation. Then, rats were intracardially perfused with 0.9% normal saline followed by 4%

paraformaldehyde (PFA) in 0.1 M PBS at pH 7.4. Spinal cord sections surrounding the T9 lesion site (approximately 1 cm long) were carefully removed from the vertebrae and fixed with 4% PFA overnight at 4°C. After dehydration, tissue was embedded in paraffin. Then, five spinal cord tissues were selected randomly from each transplanted group for cross-section and other five spinal cord tissues for sagittal section. The tissue series of slices were sectioned with 5 μm thickness and mounted on glass slides.

1) Immunohistochemistry staining

To evaluate the pathology after cell transplantation, the sagittal sectioned tissue slides were deparaffinized by xylene and rehydrated. Heat-Induced Epitope Retrieval (HIER) was performed in antigen retrieval reagent-basic solution (10 mM Tris-base, 1 mM EDTA and 0.05% tween 20, pH 9.0) for 10 min. After that, the sections were treated with 3% H_20, in PBS for 10 min. Then, the tissues were incubated in a blocking buffer (2% BSA, 5% normal goat serum, and 0.2% Triton X-100 in 0.1 M PBS) for 1h at RT.

To evaluate the survival and differentiation of the cells transplanted, immunofluorescence staining (IF) was performed. Primary antibodies were diluted in blocking buffer following B-tubulin 3 (dilution 1:100), Nuclei antibody Cy3 conjugate (HuNu; for human nuclei, dilution 1:100), microtubule-associated protein-2 (MAP2; for neuron cells, dilution 1:100), glial fibrillary acidic protein (GAP; for astrocytes; 1:100), nestin (dilution 1:100), choline acetyltransferase (ChAT; for the synthesis of acetylcholine (ACh) in cholinergic neurons, dilution 1:100), neurofilament light chain (NF-L; for neurofilament light chain, dilution 1:100) and oligodendrocyte transcription factor Anti- antibody (Olig2; for mature oligodendrocytes and myelinating Schwann cells, dilution 1:100). Tissues were incubated in primary antibodies solution at 4°C overnight. Next day, slides were stained with secondary antibodies, Alexa fluor® 488 donkey anti-mouse IgG (dilution 1:1000; InvitrogenTM, Thermo Fisher Scientific) Alexa fluor® 594 goat anti-rabbit IgG (dilution 1:250; InvitrogenTM, Thermo Fisher Scientific) and Alexa fluor® 594 goat anti-goat IgG (dilution 1:250; InvitrogenTM, Thermo Fisher Scientific) for 1h at RT and counterstained the nuclei with DAPI (dilution 1:1000). Then the tissue slides were mounted with Vectashield mounting medium. The tissue slides were observed using a fluorescence inverted microscope.

To identify brain-derived neurotrophic factor (BDNF) expression, immunoperoxidase or Immunohistochemical (IC) staining was performed. Five sets of triple adjacent slices near the midline of the spinal cord were selected and prepared for BDNF expression measurements. After that, slides incubated in blocking solution, were stained from immunoreactivity and neuroregeneration with anti-BDNF antibody (dilution 1:100) diluted in blocking buffer. Slides were incubated in primary antibodies solution at 4℃ overnight. Next day, slices were incubated with peroxidase-conjugated secondary antibody (dilution 1:100; Abcam) for 1h at RT. After washing, the peroxidase activity sites were visualized with a 3,3'-diaminobenzidine tetrachloride kit (DAB; Vector Laboratories) for 5 min. Then, slides were dehydrated and cleared in xylene. Finally, slides were mounted with a mounting medium (Bio Mount HM, Bio-Optica, Milano, Italy), covered with coverslips. Negative control tissue sections (from the MSCs group) were processed similarly but the primary antibody was omitted. Finally, immunohistochemistry tissues were observed under a fluorescence inverted microscope. BDNF expression measurements were performed using Image) software. The brown particles in the picture were regarded as positive BDNF expression.

2) Cavitation analysis

Cross-sectioned tissue slides with 5 μm thickness every 100 μm were selected by total of five slices centered over the injured epicenter of the spinal cord from each sample was obtained. Sections were deparaffinized and rehydrated. Then, the sections were incubated in 0.1% Luxol Fast Blue solution (0.1% LFB) at 60℃, overnight. Next day, tissues were differentiated in a 0.05% lithium carbonate solution (Li_2CO_3) until the tissue color turned blue for 10s followed by 70% ethyl alcohol for 30 seconds. After that, tissues were stained with Mayer's hematoxylin (Bio-Optica) for 30 sec. Then, 0.5% eosin Y (Bio-Optica) was stained for 3 min and then washed. Tissues were dehydrated and cleared in xylene. Tissues were mounted with a mounting medium (Bio Mount HM, Bio-Optica, Milano, Italy) covered with coverslips.

Five tissue slices from each sample, including the lesion center of the spinal cord, were evaluated for LFB-positive areas. The slides were observed under an inverted microscope. The integrated optical density (I.O.D.) of the myelin sheath and the number of neurons in the spinal anterior horn were obtained using Image J

software. To measure the cavitation area of each section necrotic tissue spaces, which were found in the spinal cord area, were identified as part of the injured tissue, as the cavity area (Area.cav). The area of whole spinal cord tissue was measured, as the total spinal cord area (Area.total) and calculated as percentage area cavitation (%Area.cav), which was evaluated by the formula.

$$\%Area.cav = Area.cav/Area.total \times 100\%$$

The volume of the injured spinal cord was calculated by using the Cavalieri method (Tandrup et al., 1997). This method was calculated from the area of each section multiplied by the distance between each section and then calculated to determine the percentage volume cavitation (%Volcav), which was evaluated by the formula.

$$\%VolCav = Volcav/Voltotal \times 100\%$$

4.3.6 Statistical Analysis

Statistical analysis was performed using GraphPad version 5 (GraphPad Software, San Diego, CA, USA), and data were expressed as the mean±S.D. The differences between values were determined using a one-way analysis of variance (ANOVA), followed by Tukey-Kramer Honest Significant Difference (HSD) Post hoc test to compare differences between two groups. A value of $p<0.05$ was considered sienificant with different lowercase letters. The data was plotted by GraphPad Prism version 5.

4.4 Results

4.4.1 Behavioral Tests

Behavioral tests were performed before the spinal cord injury induction, before transplantation and 8 weeks post-transplantation with BBB locomotor rating scale for 5 min (Basso et al., 1995; Lankhorst et al., 1999; Metz and Whishaw, 2002). The result showed that the average BBB locomotor rating scale (Fig. 4.2). Normal saline group showed a very low score of moving behavior at 1 week until 7 weeks post-

transplantation and significantly difference when compared with both the sham and NSs transplanted groups (p<0.05). However, BBB score of normal saline group were significantly lower than MSCs transplanted group only at 1, 3 and 5 week post-transplantation (p<0.05). In other words, the group transplanted with NSs showed spinal cord tissue was restored to be able to move similarly with normal rat (sham group) for 1 week until 8 weeks post-transplantation.

4.4.2 HIstology

1) Immunofluorescence and immunohistochemistry staining

Spinal cord tissues of all groups were examined at 8 weeks after cell transplantation with intracellular antigen-staining specific to neurons. IF was stained with B-tubulin 3, HuNu, MAP2, GAP, nestin, ChAT, NF-L, and Olig2 (Fig. 4.3-4.5). Both of MSCs and NSs transplantation groups were found HuNu stained and represent survival of the transplanted cells co-stained with neuronal antigens (B-tubulin 3) in the injured spinal cord tissue (Fig. 4.3A). However, the expression of B-tubulin 3, nestin, MAP2, NF-L, and Olig2 were not different in all groups. The expression of GAP for astrocytes showed positive expression in normal spinal cord (sham group) all around spinal cord tissue. Except in the normal saline, MSCs, and NSs transplantation groups, astrocytes were found around the injured cavities of the spinal cord tissue. Moreover, glial scar formed around the injured site of the spinal cord tissue (Fig. 4.3B). Expression of ChAT for acetylcholine as a neurotransmitter, in sham, MSCs, and NSs transplantation groups were found the expression more than in other groups (Fig. 4.4). ChAT results showed that the MSCs and NSs transplant groups have neurotransmitters similar to the sham group. In part of BDNF for immune responsibility and neuroregeneration, all groups showed greater BDNF expression in white matter (Fig. 4.6A-B). In gray matter displayed high BDNF expression in control group, before cell transplantation. Then BDNF expression still high after MSCs and normal saline transplantation for 8 weeks. Nonetheless 8 weeks after NSs transplantation, BDNF showed lower expressed than MSCs transplantation (p<0.05). From BDNF result, conclude that 8 weeks after NSs transplantation reduce the immune system response in spinal cord injury rats.

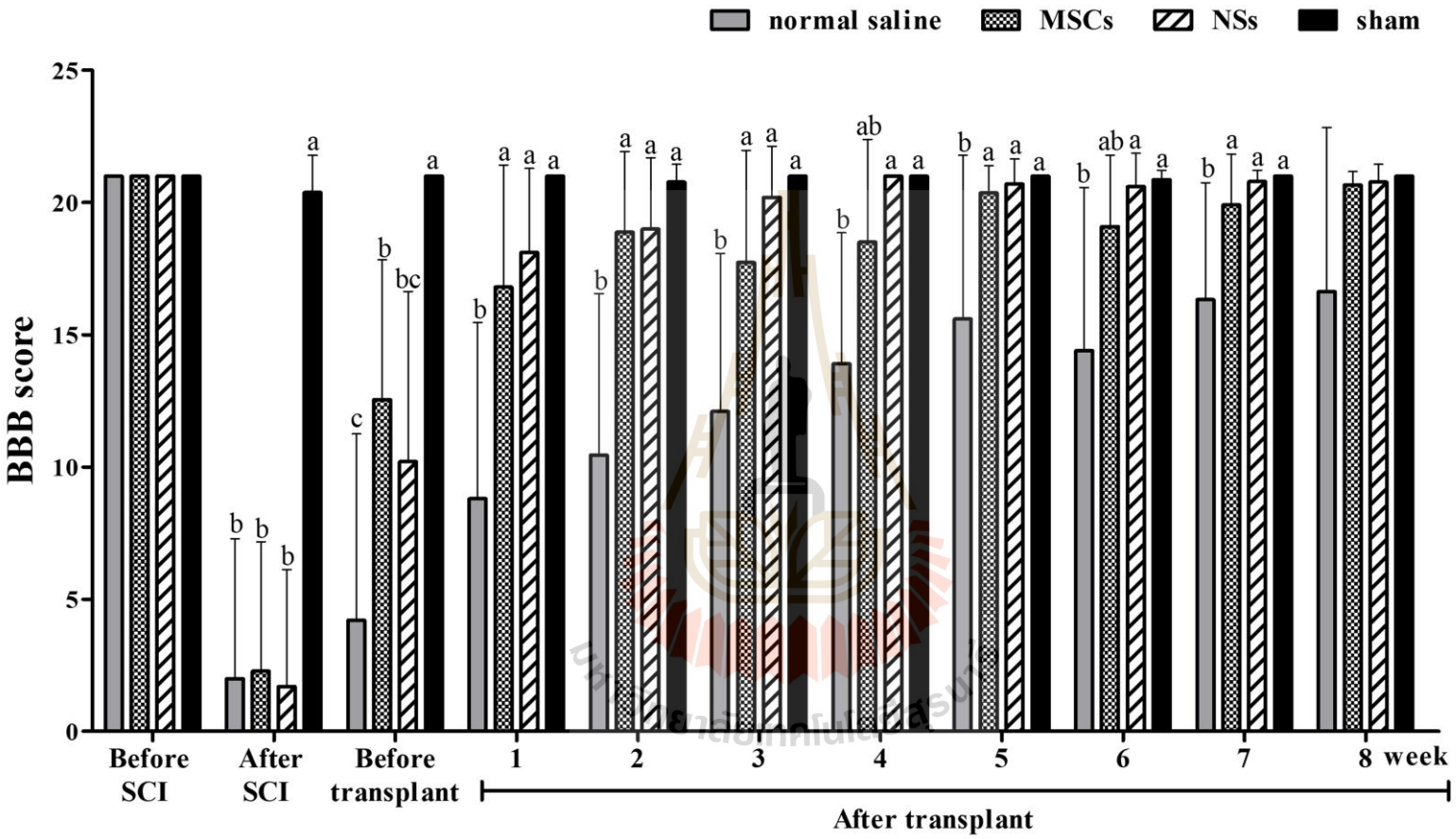

Figure 4.2 Behavioral test by BBB locomotor rating score. BBB locomotor rating score were performed before and after the SCI, after stem cell transplantation and weekly until the experiment was completed (n=8 rats/group). Data were shown as mean±S.D., with different lowercase letters, are sihnificantly different at $p<0.05$.

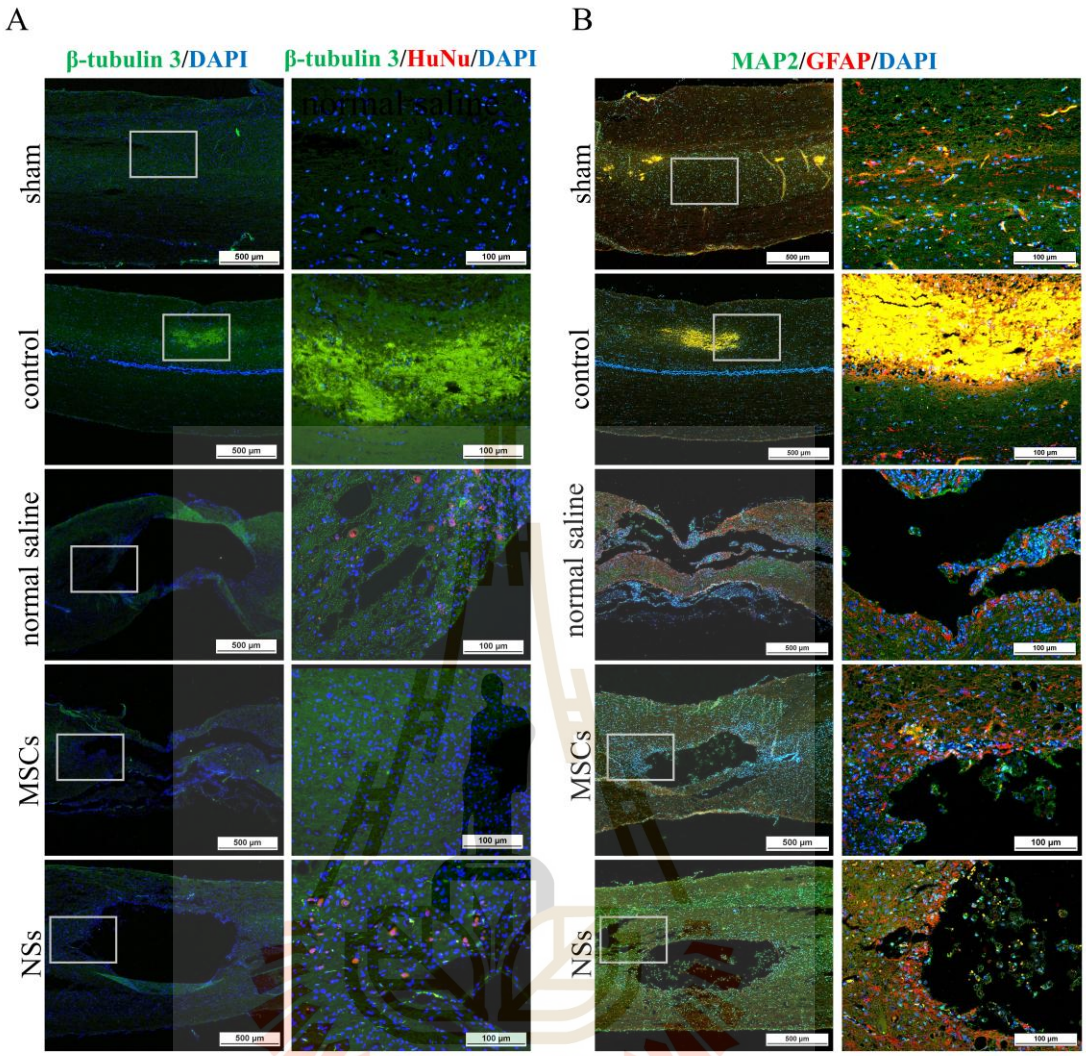

Figure 4.3 IF staining of SCI tissue 8 weeks after transplantation by β-tubulin 3, HuNu, MAP2 and GAP. (A) Images of IF staining by β-tubulin 3 (green) co-stained with HuNu (red), scale bar = 500 μm (left) and 100 μm (right). (B) Images of IF staining by MAP2 (green) co-stained with GFAP (red), scale bar = 500 μm (left) and 100 μm (right).

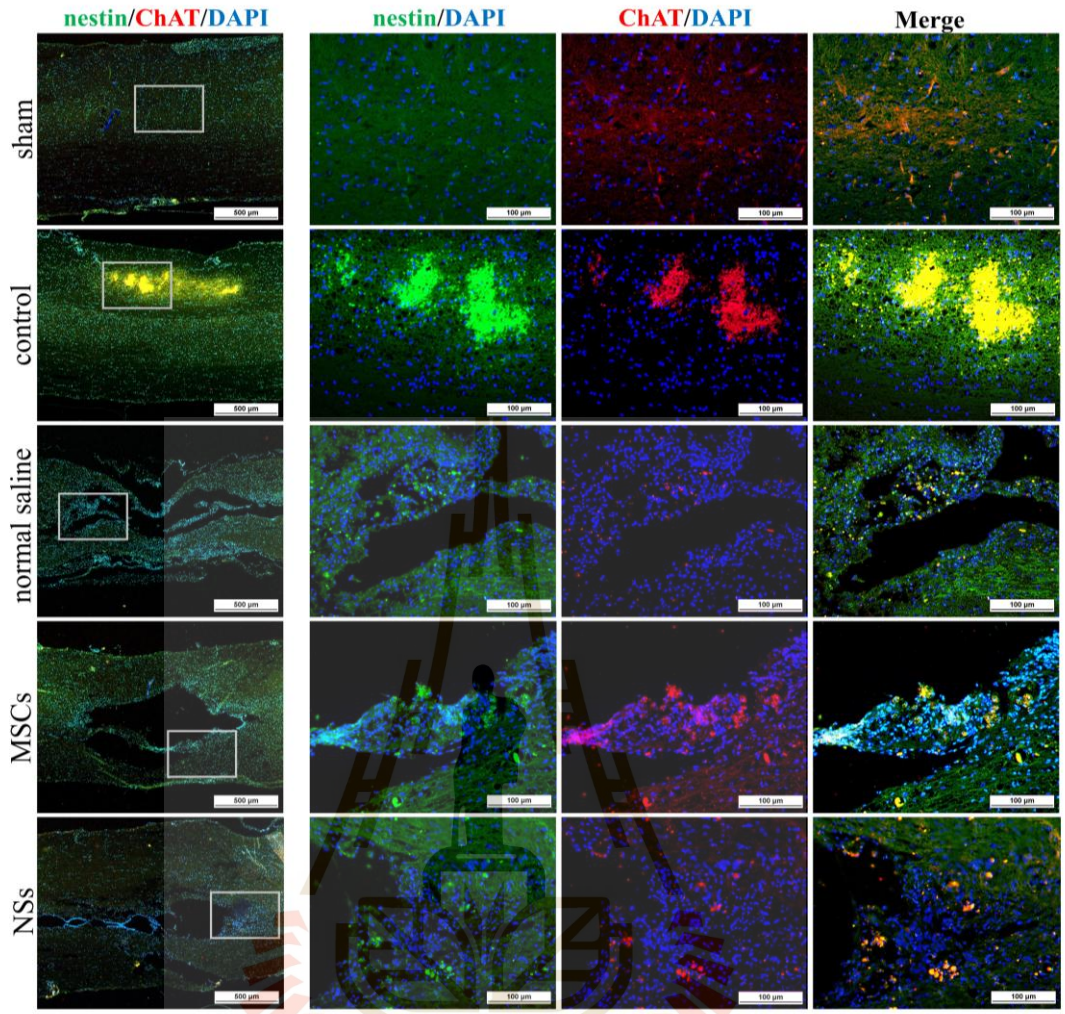

Figure 4.4 IF staining of SCI tissue 8 weeks after transplantation by nestin (green) co-stained with ChAT (red), scale bar = 500 μm (left) and 100 μm (right).

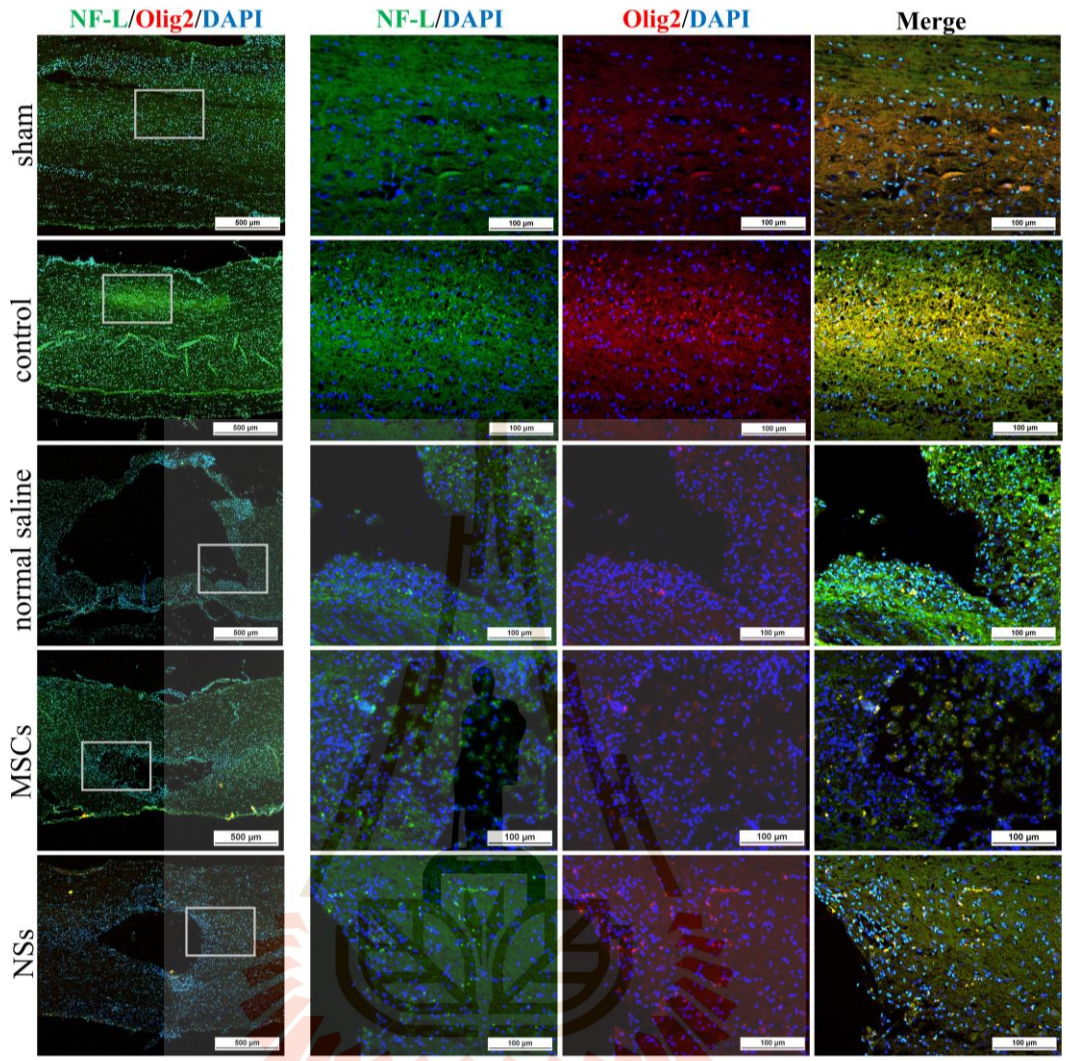

Figure 4.5 IF staining of SCI tissue 8 weeks after transplantation by NF-L (green) co-stained with Olig2 (red), scale bar = 500 μm (left) and 100 μm (right).

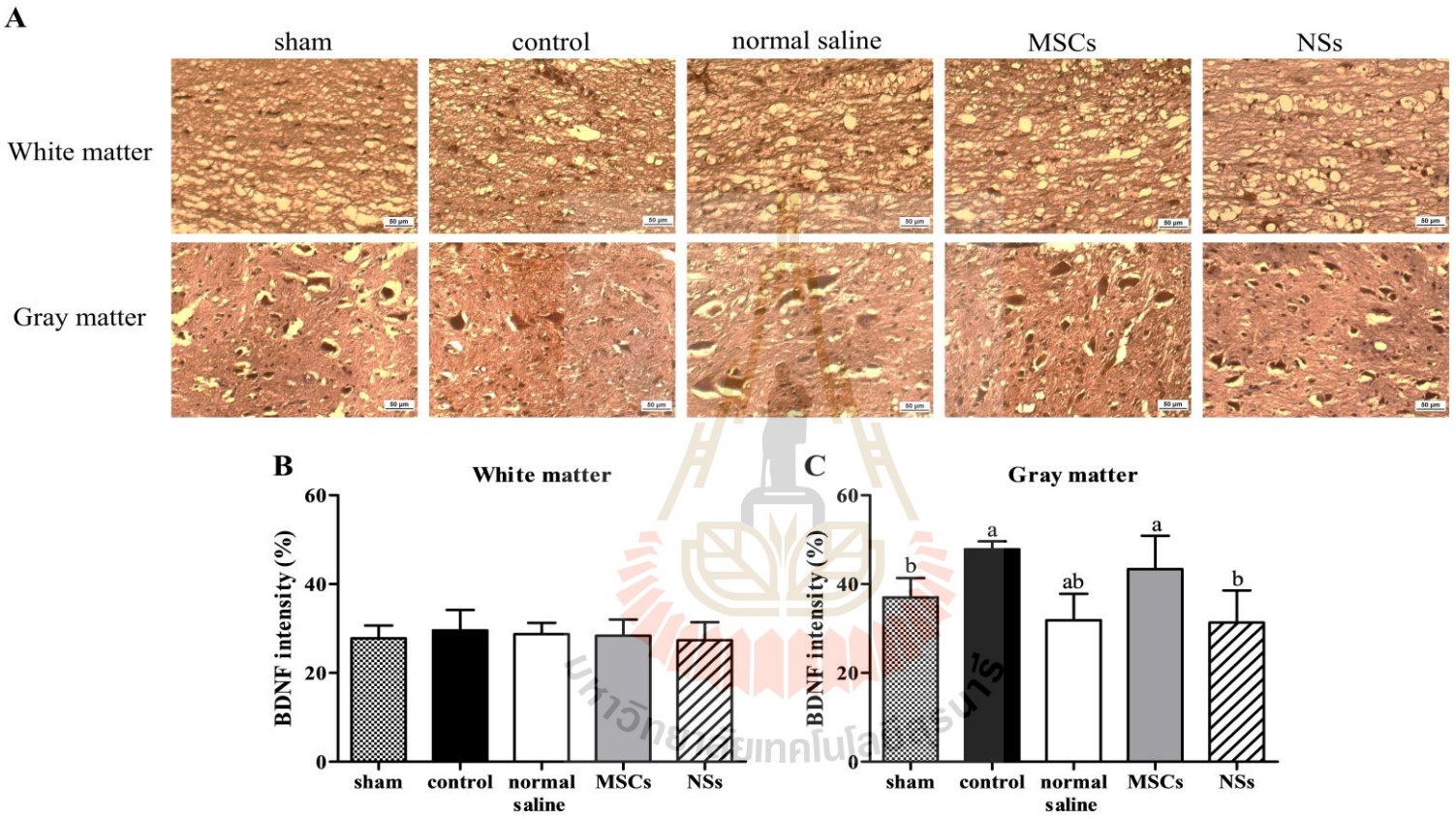

Figure 4.6 IHC staining of SCI tissue at 8 weeks after transplantation. (A) IHC staining images of BDNF at white matter and gray matter, brown particles were converged around the cavity of the spinal cord, scale bar = 50 μm. BDNF intensity results at (B) white matter and (C) gray matter of SCI tissue. Data were shown as mean ± S.D. with different lowercase letters, are significantly different at $p<0.05$.

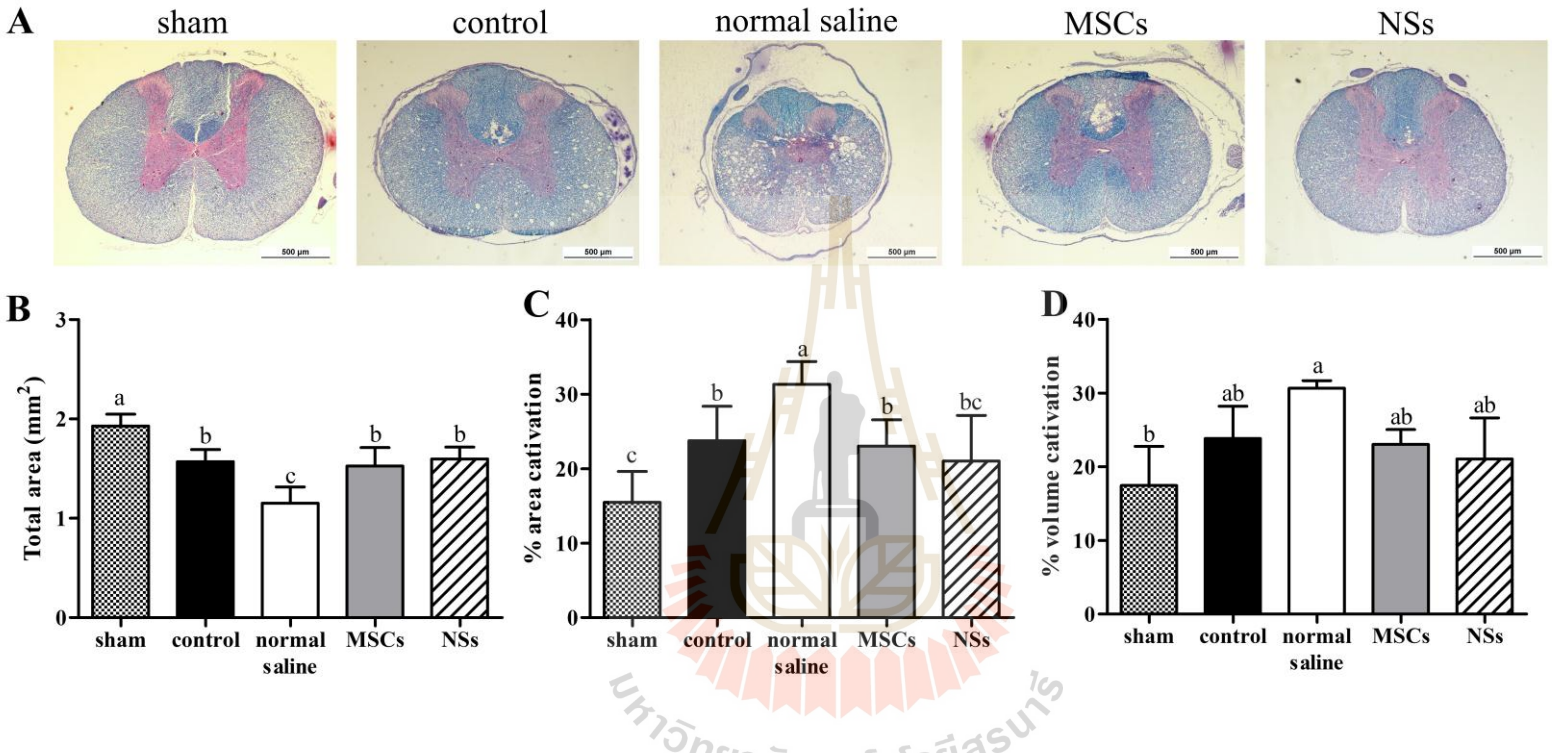

Figure 4.7 Images of LFB/H&E staining of spinal cord tissue, scale bar = 500 µm. The results of cavitation analysis after injury by LFB/H&E staining of spinal cord tissue, (B) total area of the spinal cord (mm^2), (C) percentage of area cavitation and (D) volume cavitation were showed. Data were shown as mean ± S.D. with different lowercase letters, are significantly different at $p<0.05$.

2) Cavitation analysis

The results of cavitation analysis after injury by LFB/H&E staining of spinal cord tissue (Fig. 4.7). The cavities and shrinkage of the spinal cord showed the damage of neurons from injury. Normal saline MSCs and NSs groups showed total area of the spinal cord which were was significantly smaller than sham group as shown in Fig. 4.7B (p<0.05). However, in NSs transplantation group showed small percentage of spinal cord injury space (%area cavitation) and percentage of the volume of the SCI (%volume cavitation), which no significantly difference when comparing with sham group as shown in Fig. 4.7C-D. In contrast to normal saline group percentage of area cavitation and percentage of volume cavitation showed significantly higher than sham group (p<0.05).

4.5 Discussion

In this study, hWJ-MSCs derived-NSs to transplant into SCI rat model by regulate and enhance the neuroregeneration. The NSs for *in vivo* transplantation treatment contributed to recovery in clip compression SCI model. Therefore, clip compression model be the most closely model to human SCI, which is primarily resulting from burst compression fractures and dislocation (Poon et al., 2007). Eight weeks after transplantation, the transplanted cells were differentiated into neural lineages including neurons and oligodendrocytes which were detected by specific markers including β-tubulin 3 and HuNu. Moreover nestin, ChAT, MAP2, GFAP, NF-L, and Olig2 were also evaluated in SCI tissue. Both MSCs and NSs transplantation groups were found HuNu stained for survival of the transplanted cells and neuronal antigens (β-tubulin 3) in injured spinal cord tissue. As previously reported (Hocking and Gibran, 2010; Venkatesh and Sen, 2017), MSCs transplantation to neurological disease animals also showed the recovery in nervous system. However, the expression of β-tubulin 3 (neuron cells), nestin (neural stem/progenitor cells), MAP2 (neurons), NF-L (neuron), and Olig2 (mature oligodendrocytes and myelinating Schwann cells) were found in all groups. Similarly, embryonic stem cell-derived neurospheres (ESC-NS) were transplanted to both acute and sub-acute phases of the SCI model. One to two weeks post-transplantation, specific markers including nestin,

Olig2, MAP2, astrocytes, and oligodendrocytes also were detected in spinal cord tissue (Hosseini et al., 2018). Moreover, the expression of ChAT for acetylcholine as a neurotransmitter, in sham, MSCs, and NSs transplantation groups were found the expression more than in other groups. The results showed that the MSCs and NSs transplantation groups have neurotransmitters similar to sham group (Towne et al., 2008). Furthermore, positive GFAP expression for astrocytes was spread to all the areas in normal spinal cord (sham group). Unlike in the normal saline, MSCs, and NSs transplantation group, astrocytes were found only around the injured cavities of the spinal cord tissue. GFAP expression displays in acute-subacute stage of SCI from 2 days to 2 weeks after injury which becomes a physical and chemical barrier to axonal regeneration by astrocytic scar or glia scar. Therefore, glial scar always forms around the injured site of the SCI tissue (Rowland et al., 2008; Ruff and Fehlings, 2010; Wyndaele and Wyndaele, 2006). However, astrocytes also help to recover the ionic homeostasis and the integrity of the blood-brain barrier, which helps reduce edema and immune cell invasion (Herrmann et al., 2008).

The cavities and shrinkage of the spinal cord showed the damage of neurons from injury to the results of cavitation analysis. Normal saline, control, MSCs and NSs transplantation groups showed the total area of spinal cord smaller than sham group. However, only normal saline group showed the highest percentage of area cavitation and volume cavitation, according to the highest damage area in spinal cord injury. On the other hand, the NSs transplantation group showed total area of spinal cord, percentage of area cavitation, and volume cavitation of spinal cord nearby to sham group, followed by MSCs group. The results of this study showed that MSCs and NSs transplanted groups reduced SCI space compared to the normal saline group. Preferably, NSs transplantation group might contribute to recovery in SCI. However, transplanted cells did not only recover the neuronal network but were also able to reduce inflammation, regenerate axon and synaptic and reduce glial scars which could be as cell replacement, functional multipotency, and especially stem cell regeneration (Assinck et al., 2017).

Neurotrophic factors also have been reported in neurorepair/neuroprotection and immune system response for example BDNF (Nori et al., 2017). As found in a previous report, NSs transplantation after SCI for 48 h showed immunity response

and neuroregeneration with the high BDNF antigen were detected (Ahuja et al., 2017). Similarly, previously reported (Sun et al., 2019), transplantation of MSCs from human placental cords co-cultured with neurons from the human spinal cord into SCI rat model also showed high BDNF expression. Neurotrophic factors may restore functions by cell transplantation after SCl. Neurotrophic factors secreted by stem cells to improve cell survival, cell differentiation, neuroprotection, remyelination, axonal sparing, axonal growth, neuronal survival, and synaptic plasticity with improved motor functional recovery neuroprotection which play an important role in both the PNS and CNS (Nori et al., 2017). In this study, BDNF showed greater expression in all groups at white matter of SCI tissue. On the other hand, gray matter displayed high BDNF expression in control group (before cell transplantation). Then BDNF expression still high after MSCs and normal saline transplantation for 8 weeks. Nonetheless 8 weeks after NSs transplantation, BDNF showed lower expressed than MSCs transplantation ($p<0.05$). This result related with damage cavities and shrinkage of the SCI result of after NSs transplantation. After NSs transplantation, NSs might associated with BDNF level to induce neuroprotection, remyelination, axonal sparing etc. Then 8 weeks later BDNF down regulation level was found after improved motor functional recovery as showed in the highest score of BBB locomotor tests**.**

BBB locomotor tests were performed before and after transplantation in every week. The results were found that the average BBB locomotor rating scale of the normal saline group showed very low score of moving behavior at 1 week until 7 weeks post-transplantation, and statistically significant difference was found compared with both the sham and NSs transplantation groups. This study revealed improvements in functional outcomes with treatments MSCs and NSs transplantation groups and SCI tissue were restored. Likewise, previously reported about hUC-MSCs were transplanted into SCI rats. Those rats showed the recovery movement better than without cell transplantation (Sun et al., 2019). Especially, NSs transplanted group showed spinal cord tissue was restored to be able to move similarly with normal rat (sham group) start at 1 week until 8 weeks post-transplantation. Similarly, olfactory epithelium cells from rats were induced to globose basal cells with NSs formation and then transplanted into SCI rats (contusion model). The result showed

the hindlimb motor recovery in rats at the end of the 8 weeks after transplantation (Muniswami et al., 2017).

4.6 References

Ahuja, C. S., Wilson, J. R., Nori, S., Kotter, M. R. N., Druschel, C., Curt, A., & Fehlings, M. G. (2017). Traumatic spinal cord injury. **Nature Reviews Disease Primers**, *3*, 17018. doi:10.1038/nrdp.2017.18.

Assinck, P., Duncan, G. J., Hilton, B. J., Plemel, J. R., & Tetzlaff, W. (2017). Cell transplantation therapy for spinal cord injury. **Nature Neuroscience**, *20*(5), 637-647. doi:10.1038/nn.4541.

Basso, D. M., Beattie, M. S., & Bresnahan, J. C. (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. **Journal of Neurotrauma**, *12*(1), 1-21. doi:10.1089/neu.1995.12.1.

Blight, A. R. (1991). Morphometric analysis of a model of spinal cord injury in guinea pigs, with behavioral evidence of delayed secondary pathology. **Journal of the Neurological Sciences**, *103*(2), 156-171. doi:10.1016/0022-510x(91)90159-5.

DeVivo, M. J. (1997). Causes and costs of spinal cord injury in the United States. **Spinal Cord**, *35*(12), 809-813. doi:10.1038/sj.sc.3100501.

Hagen, E. M. (2015). Acute complications of spinal cord injuries. **World Journal of Orthopedics**, *6*(1), 17-23. doi:10.5312/wjo.v6.i1.17.

Herrmann, J. E., Imura, T., Song, B., Qi, J., Ao, Y., Nguyen, T. K., . . . Sofroniew, M. V. (2008). STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. **Journal of Neuroscience**, *28*(28), 7231-7243. doi:10.1523/jneurosci.1709-08.2008.

Hocking, A. M., & Gibran, N. S. (2010). Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. **Experimental Cell Research**, *316*(14), 2213- 2219. doi:10.1016/j.yexcr.2010.05.009.

Hosseini, S. M., Sani, M., Haider, K. H., Dorvash, M., Ziaee, S. M., Karimi, A., & Namavar, M. R. (2018). Concomitant use of mesenchymal stem cells and neural stem cells for treatment of spinal cord injury: A combo cell therapy approach. **Neuroscience Letters**, *668*, 138-146. doi:10.1016/j.neulet.2018.01.008.

Lankhorst, A. J., Verzijl, M., & Hamers, F. (1999). Experimental spinal cord contusion injury: Comparison of different outcome parameters. **Neuroscience Research Communications**, *24*. doi:10.1002/(SICI)1520-6769(199905/06)24:33.0.CO;2-1.

Leung, L. (2012). Cellular therapies for treating pain associated with spinal cord injury. **Journal of Translational medicine**, *10*(1), 37. doi:10.1186/1479-5876-10-37.

Metz, G. A., & Whishaw, I. Q. (2002). Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. **Journal of Neuroscience Methods**, *115*(2), 169- 179. doi:10.1016/s0165-0270(02)00012-2.

Muniswami, D. M., Kanakasabapathy, I., & Tharion, G. (2017). Globose basal cells for spinal cord regeneration. **Neural Regeneration Research**, *12*(11), 1895-1904. doi:10.4103/1673-5374.219052.

Nori, S., Khazaei, M., Ahuja, C. S., Yokota, K., Ahlfors, J.-E., Liu, Y., . . . Fehlings, M. G. (2018). Human oligodendrogenic neural progenitor cells delivered with chondroitinase ABC facilitate functional repair of chronic spinal cord injury. **Stem Cell Reports**, *11*(6), 1433-1448. doi:10.1016/j.stemcr.2018.10.017.

Nori, S., Nakamura, M., & Okano, H. (2017). Plasticity and regeneration in the injured spinal cord after cell transplantation therapy. In (Vol. 231).

Poon, P. C., Gupta, D., Shoichet, M. S., & Tator, C. H. (2007). Clip compression model is useful for thoracic spinal cord injuries: histologic and functional correlates. **Spine**, *32*(25).

Rowland, J. W., Hawryluk, G. W., Kwon, B., & Fehlings, M. G. (2008). Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. **Neurosurgical Focus**, 25(5), E2. doi:10.3171/foc.2008.25.11.E2

Ruff, C. A., & Fehlings, M. G. (2010). Neural stem cells in regenerative medicine: bridging the gap. **Panminerva Medica**, *52*(2), 125-147.

Sun, L., Wang, F., Chen, H., Liu, D., Qu, T., Li, X., . . . Chen, Y. (2019). Co-transplantation of human umbilical cord mesenchymal stem cells and human neural stem cells improves the outcome in rats with spinal cord injury. **Cell Transplantation**, *28*(7), 893-906. doi:10.1177/0963689719844525.

Tandrup, T., Gundersen, H. J., & Jensen, E. B. (1997). The optical rotator. **Journal of Microscopy**, *186*(Pt 2), 108-120. doi:10.1046/j.1365-2818.1997.2070765.x.

Towne, C., Raoul, C., Schneider, B. L., & Aebischer, P. (2008). Systemic AAV6 delivery mediating RNA interference against SOD1: neuromuscular transduction does not alter disease progression in fALS mice. **Molecular Therapy journals**, *16*(6), 1018-1025.doi:10.1038/mt.2008.73.

Venkatesh, K., & Sen, D. (2017). Mesenchymal stem cells as a source of dopaminergic neurons: A potential cell based therapy for parkinson's disease. **Current Stem Cell Research & Therapy**, *12*(4), 326-347. doi:10.2174/1574888x12666161114122059.

Wu, W., Zhao, H., Xie, B., Liu, H., Chen, Y., Jiao, G., & Wang, H. (2011). Implanted spike wave electric stimulation promotes survival of the bone marrow mesenchymal stem cells and functional recovery in the spinal cord injured rats. **Neuroscience Letters**, *491*(1), 73-78. doi:10.1016/j.neulet.2011.01.009.

Wyndaele, M., & Wyndaele, J. J. (2006). Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? **Spinal Cord**, *44*(9), 523-529. doi:10.1038/sj.sc.3101893.

Yamazaki, K., Kawabori, M., Seki, T., & Houkin, K. (2020). Clinical Trials of Stem Cell Treatment for Spinal Cord Injury. **International Journal of Molecular Sciences**, *21*(11), 3994.

CHAPTER V
OVERALL CONCLUSION

hWJ-MSCs differentiated to NSs by neurogenesis enhancing small molecules was studied. Induction media composed of 10 μM Isx9 could induce hWJ-MSCs to be NSs by Wnt3A signaling pathway. These NSs showed both neural stem/progenitor cells by gene and protein expression such as nestin, β-catenin, β-tubulin 3 and NEUROD1. Seven days after induction in Isx9 media stem cells were selected to be used in the treatment of spinal cord injury in rat model. After transplantation, those NSs could survive and differentiate into neural lineages with neurotransmitter activity. Neurotrophic factors and axonal regeneration were also found in NS transplanted group. However, MSCs and NS transplantation group showed no different in the area of cavitation and the volume cavitation of spinal cord in injury area. Stem cell treatment from this work also improved the recovery movement in SCI rat model by BBB locomotor rating result. However, more frequent examination of the pathology and histology in different periods of the SCI model is suggested. So that more detail understanding of the mechanism of the improvement can be conclude.

APPENDIX

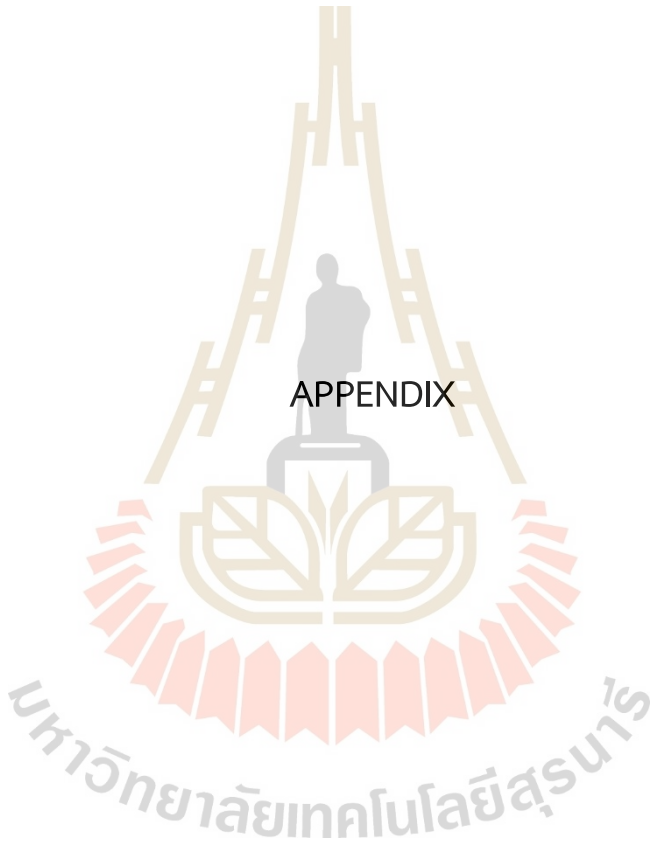

Table A1 Antibodies used for immunofluorescent, flow cytometry and western blot.

Antibodies	Companies	Catalogue No.
APC mouse anti-CD73	BioLegend	344006
APC/Cy7 mouse anti-CD90	BioLegend	328132
PE mouse anti-CD105	BioLegend	323206
PE mouse anti-CD34	Beckman Coulter	A07776
FITC mouse anti-CD45	BioLegend	368508
FITC mouse IgG, isotype Ctrl	BioLegend	400109
APC mouse IgG, isotype Ctrl	BioLegend	400120
PE mouse IgG, isotype Ctrl	BioLegend	400113
Mouse anti-nestin	Merck	MAB5326
Mouse anti-β-tubulin 3	Merck	MAB1637
Rat anti-SOX2	eBioscience	14-9811-82
Mouse anti-NEUROD1	Merck	WH0004760M1
Mouse anti-DCX	Merck	MABN707
Rabbit anti-β-catenin	Merck	06-734
Cy3 mouse anti-nuclei	Merck	MAB1281C3
Goat anti-ChAT	Merck	AB144P
Mouse anti-MAP2	Merck	MAB3418
Mouse anti-GFAP	BioLegend	644702
Mouse anti-NF-L	Merck	MAB1615
Mouse anti-Olig2	Merck	MABN50
Rabbit anti-BDNF	Merck	AB1534SP
Mouse anti-β-actin	Affinity Biosciences	T0022
Goat anti-mouse IgG HRP	Abcam	ab6789
Goat anti-rabbit IgG HRP	Abcam	ab6721
Goat anti-rat IgG HRP	Thermo Fisher Scientific	31470
Alexa fluor 488 donkey anti-mouse IgG	Thermo Fisher Scientific	A21202
Alexa fluor 594 goat anti-rabbit IgG	Thermo Fisher Scientific	A32740
Alexa fluor 594 goat anti-rat IgG	Thermo Fisher Scientific	A48264
Alexa fluor 594 donkey anti-goat IgG	Thermo Fisher Scientific	A32758

BIOGRAPHY

Miss Sirilak Somredngan was born in Bangkok, Thailand on November 2nd, 1993. She finished a Bachelor Degree (B.Sc.) in Biology from Faculty of Science, Kasetsart University (KU), Bangkok, Thailand in 2014. After that, she graduated Master Degree (M.Sc.) in Zoology from Faculty of Science, KU, in 2017. In July 2018, she started study Ph.D in Biotechnology at School of Biotechnology, Institute of Agricultural Technology, SUT. Her Ph.D study was supported by One Research One Graduate fellowships from SUT under supervision of Assoc. Prof. Dr. Rangsun Parnpai. The research topic is "Induction of human umbilical cord Wharton's jelly derived mesenchymal stem cells into neurospheres and transplantation for spinal cord injury treatment of rat model". The chapter 3 and 4 in this thesis will be published as the research article. She published five papers as follows.

Nguyen HT, Theerakittayakorn K, **Somredngan S**, Ngernsoungnern A, Ngernsoungnern P, Sritangos P, ... Parnpai R. (2022). Signaling pathways impact on induction of corneal epithelial-like cells derived from human Wharton's jelly mesenchymal stem cells. *Int. J. Mol. Sci.*, *23*(6):3078.

Theerakittayakorn K, Thi Nguyen H, Musika J, Kunkanjanawan H, Imsoonthornruksa S, **Somredngan S**, ..., Parnpai R. (2020). Differentiation induction of human stem cells for corneal epithelial regeneration. *Int. J. Mol. Sci.*, 21(21):7834. doi: 10.3390/ijms21217834.

Somredngan S, and Thong-asa W. (2017). Neurological changes in vulnerable brain areas of chronic cerebral hypoperfusion mice. *Ann. Neurosci.,* 24(4): 233-242

Somredngan S. and Thong-Asa W. (2016). Cognitive type susceptibility in ICR mice model of chronic cerebral hypoperfusion. *Biosc. Biotech.Res.Comm., 9*(3): 341-348.

Thong-Asa W, Thornmart K, Srimongkol C, **Somredngan S**, Lisanguanngam H, Tilokskulchai K. (2015). Early onset effects of mild chronic cerebral hypoperfusion on the dorsal hippocampus and white matter areas: the use of male Sprague-Dawley rats as a UCO model. *J. Neurol. Sci.*, *32*(1): 030-039.