CHAPTER II

Materials and Methods

2.1 Chemicals and reagents

Siamois polyphenols was prepared and standardized as previosly described by Desupha et al.[59] Briefly, Siamois I was prepared by using vinification and aging process as extensively described while the Siamois II was extracted from Mamoa wood as extensively described.[59] The chemical contents and quantity was measured by using HPLC (Shimadzu model LC-20, Japan) and FTIR spectrometer (FTIR AFFINITY-1,Shimadzu, Japan) . The Siamois powder was filled in 500 mg/capsule and keep in a desicator(Eureka Model K 520, Taiwan) placed in a control temperature at 23 °C until used.

2.2 Criteria of selection of volunteers

The project was approved from the Human Research Ethics Committee of Faculty of Associated Medical Sciences, Chiang Mai University (ref. no.361/2556).12 healthy donors who have blood group O were recruited and inclusion into the study was based on a questionnaire. The stem cell donors were divided in two age groups. First group was the adult age that comprised male and female who have age between 15 to 40 years and the other was elderly age that also comprise male and female with age between 50 to 70 years.

The extent of the project will be approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health (In process).15 healthy donors, the stem cell donors were divided in three age groups and each group had 5 volunteers. First group was childhood age (5-10 years), adolescent age (22- 24 years) and elderly age

(60-70 years) that comprised male and female.4 degenerative disease donor(Retinal detachment, Head injury, Stroke and CA colon) and 2 neurodegeneraive disease donor (Parkinson's disease and Multiple sclerosis).

A meeting between project coordinator and volunteers was organized. All necessary information of the study was clearly informed to the project participants. The participants signed the consent form (see in appendix section), following initial interview and physical examination.As previously mentioned, the proposed causesof Parkinson's disease and Multiple sclerosis were genetics and environmental factors whichcharacterized by age-related gradual decline in neurological function, often accompanied by neuronal death. Thus this thesis were evaluatedthe role of circulation endogenous stem cells on regeneration of neuronal tissue. The cell-based regerative neuronal tissue will comprise of (i)an elimination toxic microenvironment, (ii)restoration the tissue microenvironment to be healthy and (iii) increase in circulation endogenous stem cell in blood circulation, triggering homing and healing the damaged tissue. In order to reach the objectives, the volunteers were beenfirstly well characterized and have evidence ofadvance stage oftremor-dominant Parkinson's diseaseand relapsing–remitting Multiple scerlosis. Since it was clear that at this advance stage healing of the damaged neuronal tissuewere not be appeared in all current treatment regimens.

Parkinson patients: The inclusion criteria were selected volunteer underlying subtype oftremor-dominant Parkinson's disease (with a relative presence of other motor symptoms such as highly rigidity, bradykinesia, tremor, postural instability and dystonia) and confirm by neurological examination and medical imaging such as MRI imaging.

Multiple sclerosis patients: The inclusion criteria were selected volunteer underlying advance stage of relapsing–remitting Multiple scerlosis with the symptoms of sensory disturbance, weakness, visual loss, fatigue and bladder dysfunction. The advance stage of relapsing–remitting Multiple scerlosiswere characterized by usingmedical imaging of

MRI Brain andspine.The patients who meet the inclusion criteria must be agreed and signed the consent form before enrolling into the research project.

2.3 Protocols of treatment and blood collection

A meeting between project coordinator and volunteers were organized. All necessary information of the study were clearly informed to the project participants. The participants were signed the consent form, following initial interview, physical and neurological examination.

Blood of 10 mL were taken by the Cancer Research and Treatment Cencer Co., Ltd. Then the blood samples were immediately fill in lithium heparin tubes, gentle mixed and carry to the research laboratory.

Blood were collected before and after at different interval of treatment time using 2 capsules of Siamois I and 2 capsules of Siamois II for 4 times a day (1-5 month).

2.4 Plasma and PBMCs isolation

The blood sample tubes were centrifuged using swing-out rotor with 1800 rpm for 10 minutes. Then the plasma were separated and immediately placed in 4°C until analysis. The protein isolation process were done within 1 hr. While the buffy coat portion were aspirated and transferred into a sterile conical tube and completed the final volume of 5 mL with isotonic solution following gently mixed. A ficoll-paque solution of 5 mL were injected into the bottom of the tube, then centrifuged at 1800 rpm for 10 minutes. The PBMCs fraction were isolated and washed once using 5 mL of PBS and resuspended in 5 ml of fresh RPMI 1640 with 10% FBS. The culture flasks were placed in an incubator that control temperature to 37°C, 5% $CO_{2(g)}$ atmosphere and 95% humidity.

2.5 Flow cytometric analysis of cellular DNA contents

Cells (3×10^5) were centrifuged at 7,000 rpm for 1 minute and washed once using MD-DPBS pH 7.4 at 25°C. Afterward, 1 mL ice-cold ethanol (70% v/v) was added drop wise,

with gentle agitation and further incubation at 4 °C overnight. The cells were centrifuged at 7,000 rpm for 1 minute. The pellets of cells were discarded then 5 µl Triton x-100 (0.1% v/v), 50 µl RNase (0.2 mg/mL) and 5 µL propidium iodide (1 mg/ml; US biological, USA) were added prior to further incubation in the dark at room temperature for 30 minutes. Add 440 µl MD-DPBS vortex briefly, analyzed in a flow cytometer and fluorescence microscope (Leica, Germany).

2.6 H&E staining

H&E staining was performed for the adherent cell fraction. Briefly, the medium and suspension cells were removed and the cells were washed twice using modified-DPBS pH 7.4 at 25°C. The cells were fixed on the culture plates using 500µL formalin (4% v/v) and let incubated at room temperature for 15minutes then washed once using MD-DPBS. The membranes of cells were permeabilized using 500 mL Triton-x100 (0.1% v/v) at room temperature for 10 minutes. The cells were washed with MD-PBS following a dehydration process by adding 500 µL of ethanol with sequential incremental percentage of 70%, 95%, and absolute ethanol. Afterward, 500 mL hymatoxylin was added and placed at room temperature for 30 minutes, washed once using tab water. Add 500 µl ethanol (80% v/v) containing 1% HCl. Add 500 µl eosin and incubated at room temperature for 5 minutes and dehydration in 500 µl 70%, 95%, absolute ethanol. The cell morphology was observed under high resolution microscope.

2.7 Preparation of whole cell and plasma protein for 2D gel electrophoresis

The total protein of whole cell and plasmawere prepared as followed.Whole cells (5×10^6) weree re-suspended in solubility protein buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 40 mM DTT and 0.8%IPG buffer) for 30 minutes. Then the samples were centrifuged at 15000g, 4°C for 10 minutes using refrigerated centrifuge that fixed temperature at 4°Cand dissolved by solubility protein buffer following incubation at 4 °C under ultrasonic vibration for 30 minutes. And plasma 100 µlwere diluted with 100

µl in cooled acetone, gentle mixed prior to further overnight incubation at -20 °C.Then the samples were centrifuged at 15000g, 4°C for 10 minutes using refrigerated centrifuge that fixed temperature at 4°Cand dissolved by solubility protein buffer following incubation at 4 °C under ultrasonic vibration for 30 minutes. The total protein of whole cell and plasmawere determined by using Bradford assay.

2.8 Two-dimensional gel electrophoresis

Protein samples 1 mg/gel were mixed with solubility protein buffer 7 M urea, 2 M thiourea, 4 % CHAPS, 40 mM DTT, 0.8%IPG buffer and trace of bromophenol bluefor the pH range 3-10, to obtain a final volume of 400 ul.Focusing were passive rehydrate at 20°C, 12 hr. and IEF protocol at 20°C, 12hr. For a total of 200-8000V. For the second dimension, IPG gel strips were equilibrated for 30 min in equilibrium buffer 7M urea, 375mM Tris, 10% glycerol, 3% SDS, containing 52.8mM DTT and 142mM IAA. Proteins were separated on freshly prepared 12% SDS-polyacrylamide gels with a constant current of 50mA for 6 hr. Gradient gels were prepared in a cold chamber using a gradient maker connected to a peristaltic at 15°C. Proteins were visualized using coomassie blue stainingand analysis protein spot by densitometer scanner GS900 (Biorad). The proteins corresponding to genes (consulting the www.pantherrdb.org and www.phosphosite.org) that govern the repair and regeneration processes was obtained byspot gel excision following elution and digestionthen analyzed by LC-MS.

2.9 Electrospun of PVDF 3D-nanofibrous scaffold

The in house made 3D-nanofibrous PVDF membrane was fabricated using electrospinning technique as extensive described by Choi (Choi, Lee et al. 2004). Polyvinylidene fluoride) the so-called PVDF (Solvay Co., France) of 25 wt.% was dissolved in *N*,*N*-Dimethylacetamide (DMAA) (Aldrich). Syringe having metal needle (1 mm of diameter) was used as the solution reservoir. A drum shaped counter electrode was located opposite to the reservoir. The fibers were collected on the tubular layer at

the winding drum of 30 rpm. The distance between the capillary tip and the collector (tip-to-collector distance, TCD) was 15 cm and the applied voltage was 10 kV. A thin polymer fiber is deposited on the collector. The thickness of the fibers was expressed as the spinning time such as 1, 2 and 3 hrs. Electrospun fibers are collected as mix morphology of a non-woven and aligned form. Morphology of the electrospun PVDF nanofiber was observed with scanning electron microscope (SEM) of JEOL model JSM-5410LV. The circular and square 3D-nanofibrous PVDF scaffolds with varied thickness were constructed by fixing the membrane on the structure of polyethylene plastic.

2.10 Video-optical imaging of circulating endogenous stem cell behavior on a 3D nanofibrous scaffold

The 3D-nanofibrous PVDF scaffolds were disinfected by purging in 70% alcohol for 30 minutes and were washed using sterile phosphate buffer pH 7.4 at room temperature in biohazard cabinet. The scaffolds were UV-C irradiated for 1 hr then immersing into sterile RPMI-1640 medium and let incubated at 37 °C for 24 hrs in a $CO_{2(g)}$ incubator. The scaffolds were transferred to 6-well plates. PBMCs (60 µL of 10^7 cells) were seeded onto the scaffolds and further incubation at 37 °C for 24 hrs in a $CO_{2(g)}$ incubator, then 4 mL of fresh RPMI 1640 medium were added into the wells and further incubation for 72 hrs. Then the scaffolds were transferred into new 6-well plates and completed with 4 mL of RPMI 1640, incubated at 37 °C in a $CO_{2(g)}$-incubator. The culture were maintained by changing the culture medium once a week and their behavior were monitored using video-optical imaging.

2.11 Scanning electron microscopy sample preparation

The scaffolds were firstly fixed by immersing 2.5% glutaraldehyde in 0.1Mcacodylate buffer, pH 7.4 for 1 hours at room temperature or at 4° C (in refrigerator) overnight. They were washed 2 times in 0.1 M cacodylate buffer pH 7.4; each 10 minute duration. Then the second fixation was performed by immersing the scaffolds in 1% osmium tetroxide

(aqueous) pH 7.4 for 2 hour at room temperature and in a light tight container. The scaffolds were again washed 2 times in 0.1 M cacodylate buffer pH 7.4; each 10 minute duration. The scaffolds were dehydrated by sequentially immersing as follows: 1 x 10 min. in 30% ethanol, 1 x 10 min. in 50% ethanol, 1 x 10 min. in 70% ethanol, 1 x 10 min. in 80% ethanol, 1 x 10 min. in 90% ethanol, 1 x 10 min. in 100% ethanol. The scaffolds were then submitted to perform critical point dry which is an automated process takes approximately 40 minutes. The scaffolds were mounted onto metal stub with double sided carbon tape. Finally, a thin layer of gold and palladium were coated over the scaffolds using an automated sputter coater.

2.12 Motor symptom evaluation

Average scores, pre-post treatment scores were calculated. Motor symptom were calculated as the sum of all scores. Last day, scored were chosen as the endpoint for calculating clinical symptom. The grade were preferred in the studies, the grade correlated with the clinical neurological examination of the patient.

Parkinson disease: The grade were preferred 0–5 grade in the studies, the grade correlated with the clinical neurological examination of the patient. The grade system represents the sum of rigidity, bradykinesia, tremor, postural instability and dystonia.

In the 0–5 grade, the modified Hoehn and Yahr scale is as follows.[78]

Grade 0 No signs of disease

Grade 1 Unilateral disease

Grade 2 bilateral diseases, with no impairment of balance

Grade 3 Mild to moderate bilateral disease

Grade 4 Severe disability; still able to walk or stand unassisted

Grade 5Wheelchair-bound or bedridden unless aided

Multiple sclerosis: The grade were preferred 0–10 grade in the studies, the grade correlated with the clinical neurological examination of the patient The grade system

represents the sum of Sensory disturbance, Weakness, Visual loss, Fatigue and Bladder dysfunction.

In the 0-10 grade, the modified Kurtzke JF is as follows.[79]

Grade 0 Normal neurologic exam

Grade 1 No disability

Grade 2 Disability is minimal

Grade 3 Disability is mild to moderate

Grade 4 Disability is moderate

Grade 5 Increasing limitation in ability to walk

Grade 6 Walking assistance is needed

Grade 7 Confined to wheelchair

Grade 8 Confined to bed or chair

Grade 9 Completely dependent

Grade 10Death due to MS

2.13 MRI imaging

The MRI scan were used to compare images of lesions before and after treatment with Siamois polyphenols (A schematic timeline of the study is provided in figure 7). Image acquisition all MRI were obtained with a 1.5-T MRI scanner (Phillips)

Parkinson disease: MRI brain with thin slide in midbrain.

Technique of MRI brain: Axial view: T1W, T2W, FLAIR and DWl. Sagittal view: T2W. Coronal view: T2W (GRE)

Multiple sclerosis: MRI brain and spine.

Technique of MRI brain: Axial view: T1W, T2W, FLAIR and DWl, ADC map, T1W+Gd. Sagittal view: T2W+Gd. Coronal view: GRE-T2W, T1W+Gd

Technique of MRI whole spine:Axial view: T1W, T2W, T1W+Gd+Fs. Sagittal view: T1W, T2W, T1W+Gd+FS. Coronal view: T2W, T1W+Gd+Fs