CHAPTER II

MATERIALS AND METHODS

2.1 Collection of human peripheral blood mononucleated cell (PBMCs)

Blood samples (5 ml) obtained from healthy adults were collected (AMS Clinical Service Center, Faculty of Associated Medical Sciences, Chiang Mai University) in Violet-topped Vacutainer tubes containing EDTA. The samples were mixed immediately by turning them upside down 3-5 times to ensure a mixture with the anticoagulant. Samples were immediately transferred to the laboratory. PBMCs can be isolated from whole blood samples using different density gradient centrifugation procedures. Anticoagulated whole blood is layered over the separating medium (ficoll hypaque (Lymphoprep™, Norway)). The samples were centrifuged at 300 x g for 5 minutes. At the end of the centrifugation step, the following layers were visually observed from top to bottom: plasma/platelets, PBMCs, separating medium and erythrocytes / granulocytes (Figure 2.1). The PBMCs layer was then removed and lysised with Red blood cell (RBC) lysing solution. PBMCs were then washed with balanced salt phosphate buffer solution (PBS) pH 7.4 to eradicate some contaminants.

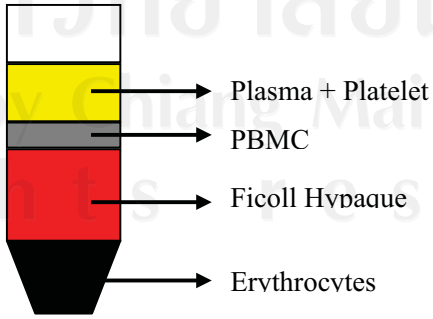

Figure 2.1 PBMCs separation by Ficoll hypaque gradient centrifugation

2.2 Numeration of $CD34^+$ cells from PBMCs cultures

The surface proteins, CD34 will be used for characterizing stem. PBMCs (10^6 cells) were centrifuged at 7000 rpm for 1 minute and washed once using phosphate buffer pH 7.4 at 25°C. The successive addition of anti-$CD34^+$ - FITC (10 μL) following 100 μL PBS pH 7.4 at 37°C was added into the cell pellets. The suspension of cells was incubated in the dark at 37°C for 15 minutes. Finally, 400 μL PBS was added and the cells were analyzed using flow cytometer and fluorescence microscope.

2.3 Expansion of adult PBSCs in conventional culture

Cells (10^6cells/mL) were cultured in 25 cm^2 cultured flask or 24-well plates with RPMI1640 (Gibco,USA) or MEM (Gibco,USA) media supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (PAA, Austria) at 37 °C in a humidified incubator of 95% humidified. The cell morphology were observed and micrograph under inverted light microscope at 24 hr of interval time. The cells were counted using haemocytometer. Cultures were maintained at initial density of 10^7 cells/mL.

2.4 Evaluation of adult PBSCs differentiation using $CD34^+$ and $CD11b^+$.

The surface proteins including $CD34^+$ and $CD11b^+$ were used for characterizing the PBSCs differentiation. Cells (10^6) were centrifuged at 7000 rpm for 1 minute and washed once using phosphate buffer pH 7.4 at 25°C. The successive addition of anti-$CD34^+$ -FITC (10 μL) or $CD11b^+$-FITC following 100 μL PBS pH 7.4 at 37°C was added into the cell pellets. The suspension of cells was incubated in the dark at 37°C for

15 minutes. Finally, 400 μL PBS was added and the cells were analyzed using flow cytometer (Beckman Coulter) and fluorescence microscope (Leica, Germany).

2.5 Flow cytometric analysis of cellular DNA contents.

Cells (10^6cells/mL) were cultured in 24-well plates with RPMI 1640 (Gibco, USA) or MEM (Gibco, USA) media supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (PAA, Austria) at 37°C in a humidified incubator of 95% humidified. The cell morphology were observed and micrograph under inverted light microscope at 24 hr of interval time. A total of cells were centrifuged and washed once using phosphate buffer saline (PBS). The cell pellets were resuspended in 300 mL PBS then 700 mL ice-cold ethanol was added, dropwise, with gentle agitation and future incubation at 4°C overnight. The cells were centrifuged at 14,000*g*. Aspirate the supernatant and resuspend cells in 500 μl of PBS containing 0.1% Triton X-100, 0.2 mg/ml RNase A, and were then stained with 10 mg/mL propidium iodide (USbiological, USA) then let incubated at 37°C for 30 minutes. The cell cycle distribution was analyzed for 10,000 collected cells by a flow cytometer (Beckmann Coulter).

2.6 Electrospun of PVDF 3D-nanofibrous scaffold

The in house made 3D-nanofibrous PVDF membrane was fabricated using electrospinning technique as applied from Chanunpanich (Chanunpanich, Lee, & Byun, 2008). Polyvinylidene fluoride) the so-called PVDF (Kynar 441) of 19 wt% was dissolved in *N,N*-Dimethylacetamide (DMAA) (Fluka) and acetone (4:6 wt%). Syringe having metal needle (G22) was used as the solution reservoir. A drum shaped counter

electrode was located opposite to the reservoir. The fibers were collected on manmade plastic plate at the winding drum of 52 m/s. The distance between the capillary tip and the collector (tip-to-collector distance, TCD) was 10 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 hr. 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.

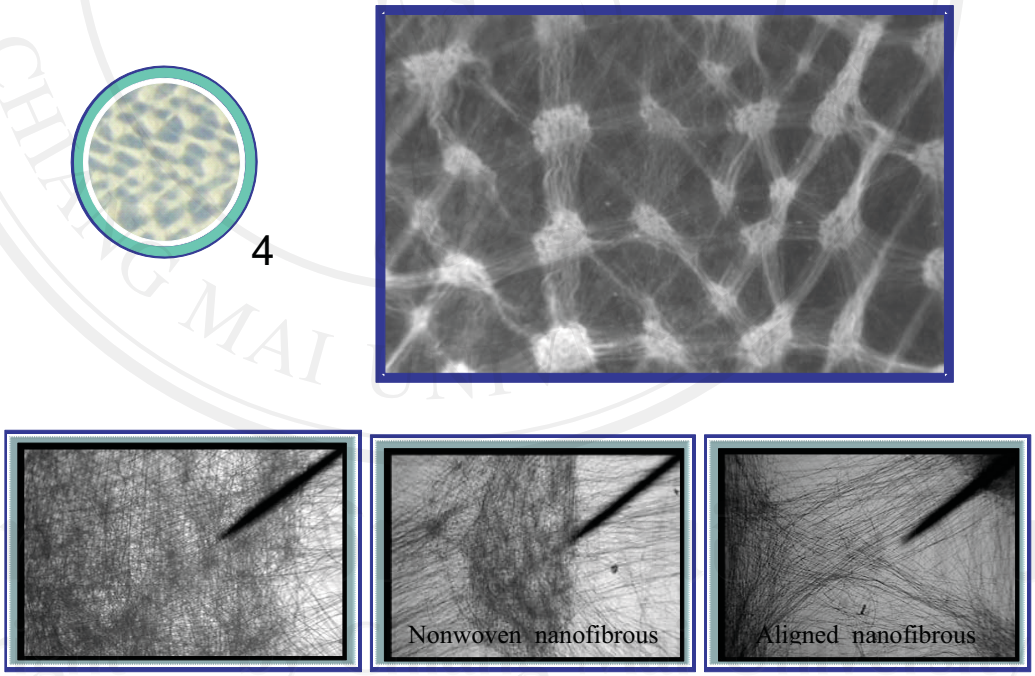

Figure 2.2 Mix morphology of 3D-nanofibrous scaffold of nonwoven and aligned nanofibrous

2.7 PBSCs growth and differentiation on PVDF 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-incubator. The scaffolds were transferred to 6-well plates. PBMCs (50 μL of 3×10^6 cells) were seeded onto the scaffolds and further incubation at 37°C for 24 hrs in a CO_2-incubator, then 4 mL of fresh RPMI 1640 medium was 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-incubator. The culture was maintained by changing the culture medium once a week.

2.8 Fluorescence microscopy

PBMCs culture on scaffold at 60 days after culture was washed with PBS buffer and co-staining with anti-CD34 conjugated. Then incubated for 30 minutes in the dark and washed with PBS buffer.

2.9 Scanning electron microscopy sample preparation

The scaffolds were firstly fixed by immersing 3% glutaraldehyde in 0.1M Cacodylate buffer, pH 7.4 for 4 hours at room temperature or at 4°C (in refrigerator) overnight. They were washed 3 times in 0.1 M cacodylate buffer pH 7.4; each 15 minute duration. Then the second fixation was performed by immersing the scaffolds in 1% osmium tetroxide (aqueous) pH 7.4 for 3 hour at room temperature and in a light tight

container. The scaffolds were again washed 3 times in 0.1 M cacodylate buffer pH 7.4; each 5 minute duration. The scaffolds were dehydrated by sequentially immersing as follows: 1 x 10 min. in 25% ethanol, 1 x 10 min. in 50% ethanol, 1 x 10 min. in 70% ethanol, 1 x 10 min. in 85% ethanol, 1 x 10 min. in 95% ethanol, 2 x 10 min. in 100% ethanol, 1 x 10 min. in 100% ethanol (EM grade). 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.