CHAPTER IV
MATERIAL AND METHODS

4.1 Material

4.1.1 Chemicals

The chemical reagents for cell culture, MTT assay, fluorescence assay and western blot analysis are listed in Table 4.1. All other chemicals were analytical grade.

4.1.2 Instruments

- Synergy HT multi-detection microplate reader (Bio-Tek Instruments, Winooski, VT)

- Phase constant light microscopy (Nikon Eclipse TE2000-s) attached with digital camera (CANON Powershot A710IS)

- Western blot kit (Bio-Rad Laboratories, Inc., USA)

4.1 List of Chemical reagents

Chemical	Supplier
Beta-actin mouse mAb	Cell Signaling Technology (Beverly, MA)
Biotynylated protein ladder	Cell Signaling Technology (Beverly, MA)
Calcein, AM	Invitrogen (CA, USA)
Deferiprone	The Government Pharmaceutical Organization, Bangkok, Thailand
2', 7'- dichlorodihydrofluorescein diacetate (DCFH-DA)	Molecular probe (Eugene, OR)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphentetrazolium bromide (MTT)	Sigma-Aldrich (St. Louis, MO, USA)
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco BRL (Grand Island, NY)
Dulbecco's Modified Eagle's Medium - low glucose (DMEM(-)phenol red)	Sigma-Aldrich (St. Louis, MO, USA)
Fetal Bovine Serum (FBS)	PAA Lab (Queensland, Australia)
Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG	Cell Signaling Technology (Beverly, MA)
Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG	Cell Signaling Technology (Beverly, MA)
iNOS rabbit mAb	Santa cruz Biotechnology (Texas, USA)
Lipopolysaccharide (LPS, Escherichia coli serotype 026:B6)	Sigma-Aldrich (St. Louis, MO, USA)
p-p38 MAPK rabbit mAb	Cell Signaling Technology (Beverly, MA)
p-38 MAPK rabbit Ab	Cell Signaling Technology (Beverly, MA)
Tryphan Blue	Gibco BRL (Grand Island, NY)
Trypsin-EDTA	Gibco BRL (Grand Island, NY)

4.2 Methods

4.2.1 Viruses

Japanese encephalitis virus (JEV, Nakayama strain), dengue-2 (DENV-2, 16681 strain), dengue-4 (DENV-4, H241 strain) and Culex flavivirus (CxFV) were used for infection experiment throughout this study.

4.2.2 Cell culture

Highly Aggressive Proliferating Immortalized (HAPI) cells were generously provided by Prof. James R. Conor (Department of Neuroscience and Anatomy, Hershey, Medical Center, Hershey, PA). HAPI cells exhibit various microglia characteristics including phagocytosis, production of cytokines, ROS and NO [15].

HAPI cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator under 5% CO_2 and 95% air.

HAPI cells were seeded at density $5x10^5$cells/ml in 75 cm^2 flask. Fresh medium was replaced every 2 days. In subcultivation process, the medium was removed and washed 2 times with 4 ml 1X phosphate-buffered saline (1X PBS), following by addition of 2.5 ml of 0.125% of trypsin-EDTA and 5 ml of DMEM supplemented with 2.5% FBS. Then the sample was centrifuged 2 times at 200 g for 5 minutes at room temperature. The supernatant was removed and added DMEM supplemented with 2.5% FBS. Then HAPI $5x10^5$cells were harvested and DMEM supplemented with 2.5% FBS was added in 75 cm^2 flask.

4.2.3 Virus infection [15]

HAPI cells (at density 10^4 cells/well for 96 wells plate or $2x10^5$ cells/well for 6 wells plate) were seeded and maintained in free-phenol red DMEM supplemented with 2.5% FBS at 37°C in a humidified incubator under 5% CO_2 and 95% air for 24 hours before challenging with flavivirus (JEV, DENV-2, DENV-4 and CxFV) at multiplicity of infection (MOI) of 0.01 and 0.1. Infection time was 2 hours at

37°C with constant agitation. After this period, medium was removed and then replaced with fresh medium. At 6, 24, 48 and 72 hours post-infection, medium was collected for determination of ROS production, oxidative products, IL-1β, IL-6, nitric oxide and virus titer. The cells were washed with 1X PBS before performed intracellular ROS production and cell viability.

4.2.4 Intracellular ROS production measurement [52]

At indicated time (6, 24, 48 and 72 hours post infection), medium was removed and the cells were washed with 1X PBS twice. Then 20 μM of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) in free phenol-red DMEM without fetal bovine serum was added an incubated for 30 minutes in the dark at 37°C before washed twice with 1X PBS and added free phenol-red without fetal bovine serum. Fluorescence intensity was analyzed in immediately by using fluorescence microplate reader at excitation wavelength 485 nm and emission wavelength 528 nm.

4.2.5 Cell viability

Cell viability was determined by the quantitative colorimetric of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphentetrazolium bromide) assay. Following ROS determination, medium was removed and the cells were incubated with a solution of 1 mg/ml MTT for 3 hours at 37°C and 5% CO_2. After that, MTT reagent was removed and formazan crystals were solubilized with 100 μl DMSO, and measured the absorbance at 562 nm and 630 nm via microplate reader. After subtraction of background, cell viability was expressed as a percentage related to control that was designed as 100%.

4.2.6 Nitric oxide (NO) production [53]

NO production was determined in medium by mean of fluorometric measurement of nitrite/nitrate by 2,3-diaminonaphthalene (2,3-DAN) as described by Nussler et al., 2006. Briefly, a 150 μl of medium sample was added with 2,3-DAN in HCl and was incubated for 5 minutes at 30°C in dark. Then 3.0 N NaOH was added, the mixture was immediately measured the fluorescence at excitation wavelength 365

nm and emission wavelength 410 nm. Sodium nitrite solution was used as standard solution.

4.2.7 Interleukin-6 (IL-6) / Interleukin-1β (IL-1β)

IL-6 and IL-1β were determined in the medium using Rat IL-6 and Rat IL-1β ELISA kits (Thermo Scientific, Rockford IL) according to the manufacture's instruction. Samples were done in duplicated in each condition.

4.2.8 Western blot analysis

HAPI cells were cultured on 6 wells plates at density of $2x10^5$ cells/well. The cells were challenged with 4 viruses and LPS according to experimental design. Proteins were collected after cell lysis with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% TritonX-100, 0.1% sodium deoxycholate, 5mM EDTA, 30 mM Na_2HPO_4, 50 mM NaF mixed with 1% protease inhibitor cocktail and/or 0.5% $NaVO_4$). The protein concentration was determined by Lowry's method by using BSA as a standard.

A 40 μg of protein sample was electrophoresed on 12.5% SDS-polyacrylamide gel using 1X running buffer 1.5M Tris-HCl pH 8.8 and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in 1X PBST or 5% bovine serum albumin (BSA) in 1X PBST. After the membrane was blocked, the membrane was incubated with the primary antibody overnight at 4°C. Afterward the membrane was rinsed with 1X PBST 5 mins 3 times and followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or HRP-conjugated goat anti-mouse secondary antibodies for 1 hour at room temperature. After that, the membrane was rinsed with 1X PBST for 3times of 5 minutes. Finally, the membrane was visualized by enhaced chemilunescence using ECL plus^{TM} wester blotting detection reagents and exposed on Hyperfilm ECL nitrocellulose membrane. The immunoblot bands were quantified by measured the intensity of each band using ImageJ software (ImageJ software, Marylang, DC, USA). β-actin was used as internal control and the result were represent as a fold control.

4.2.9 Statistical analysis of data

All experiment were performed at least 3 independent experiments. Data were expressed as the mean ± SEM of experiment in which triplicates samples were performed. The differences of all parameters were analyzed with one-way ANOVA followed by Bonferroni test using GraphPad Prism 5 software (version 5; GraphPad, San Diego, CA, USA) and considered significant when $p<0.05$.

4.2.10 Experimental design

Experiment 1: Study the effects of t-BuOOH and LPS induced ROS production in microglia cells.

HAPI cells were cultured in medium supplemented with 2.5% FBS at density of $1x10^4$ cells/well on 96 well plates. The cells were incubated in medium supplement with various concentrations of t-BuOOH (1, 10, 100, 150 and 200 μM) for 1 hour or 1 μg/ml of LPS for 24 hours before cell viability and intracellular ROS production measurement were performed. The experimental flow chart is shown in Figure 4.1

Experiment 2: Study the effects of flavivirus induced ROS production in microglia cells

HAPI cells were cultured as described previously. HAPI cells were challenged with flavivirus (JEV, CxFV, DENV-2 and DENV-4) at multiplicity of infection (MOI) of 0.01 and 0.1 or 1 μg/ml of LPS for 2 hours absorption period. Cell viability, ROS determination was performed at 6, 24, 48 and 72 hours. The medium was collected for Nitric oxide and IL-1β determination. The experimental flow chart is shown in Figure 4.2

Experiment 3: Study the response of HAPI cells followed the flavivirus infection

To investigate the target molecular signaling pathway in response to flavivirus infection, the major signaling pathway that involved including, caspase mediated apoptotic pathway and inducible nitric oxide synthase (iNOS) pathway were studied as the following experiment. HAPI cells were cultured on 6 wells plates at density of $2x10^5$ cells/well. HAPI cells were challenged with flavivirus (JEV, CxFV, DENV-2 and DENV-4) at multiplicity of infection (MOI) of 0.01 and 0.1 for 2 hours

absorption period. Western blot analysis was performed as mention earlier. The experimental flow chart is shown in Figure 4.3

4.2.10 Experimental design

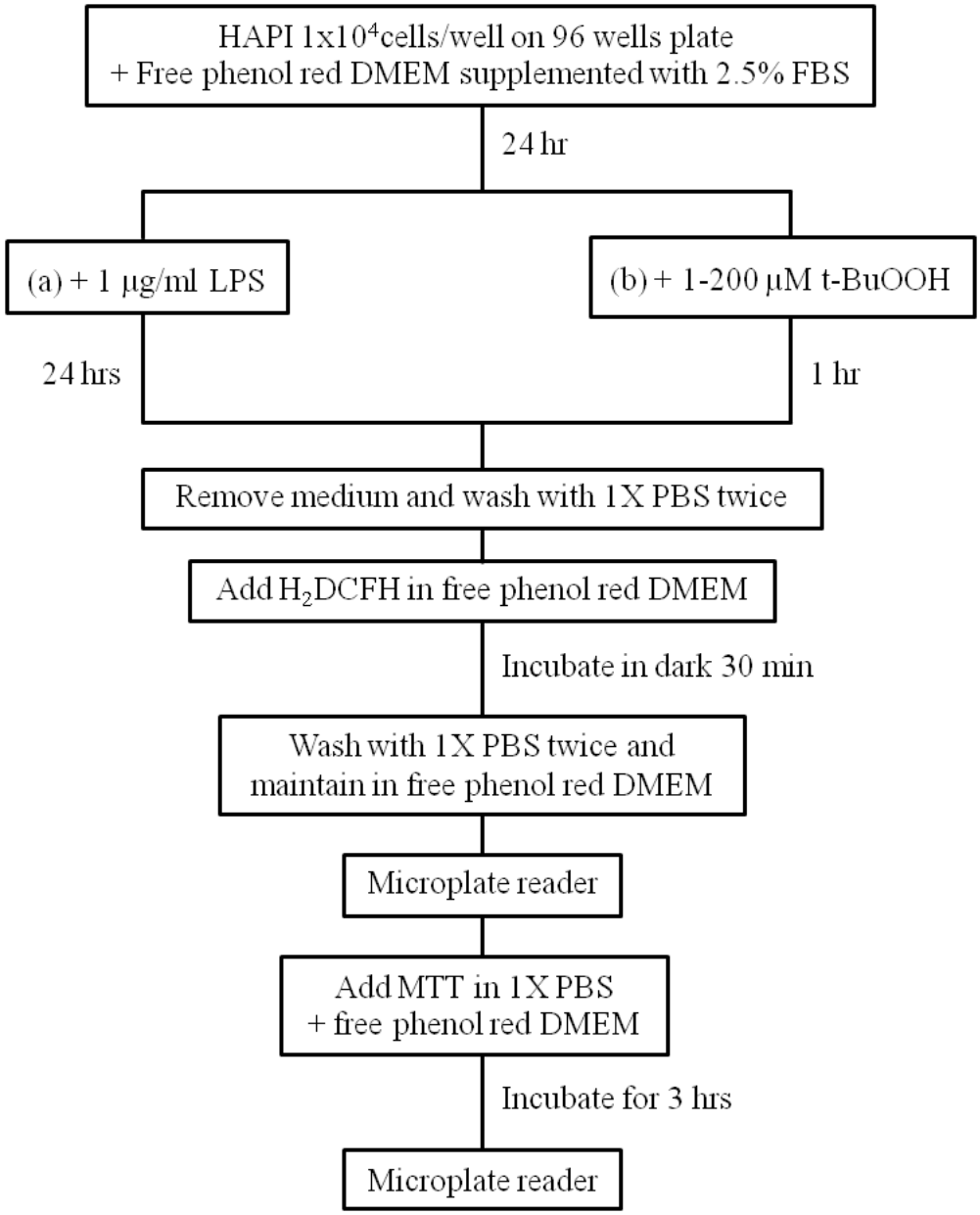

Figure 4.1 Experimental flow chart study the effects of LPS and t-BuOOH in HAPI cells

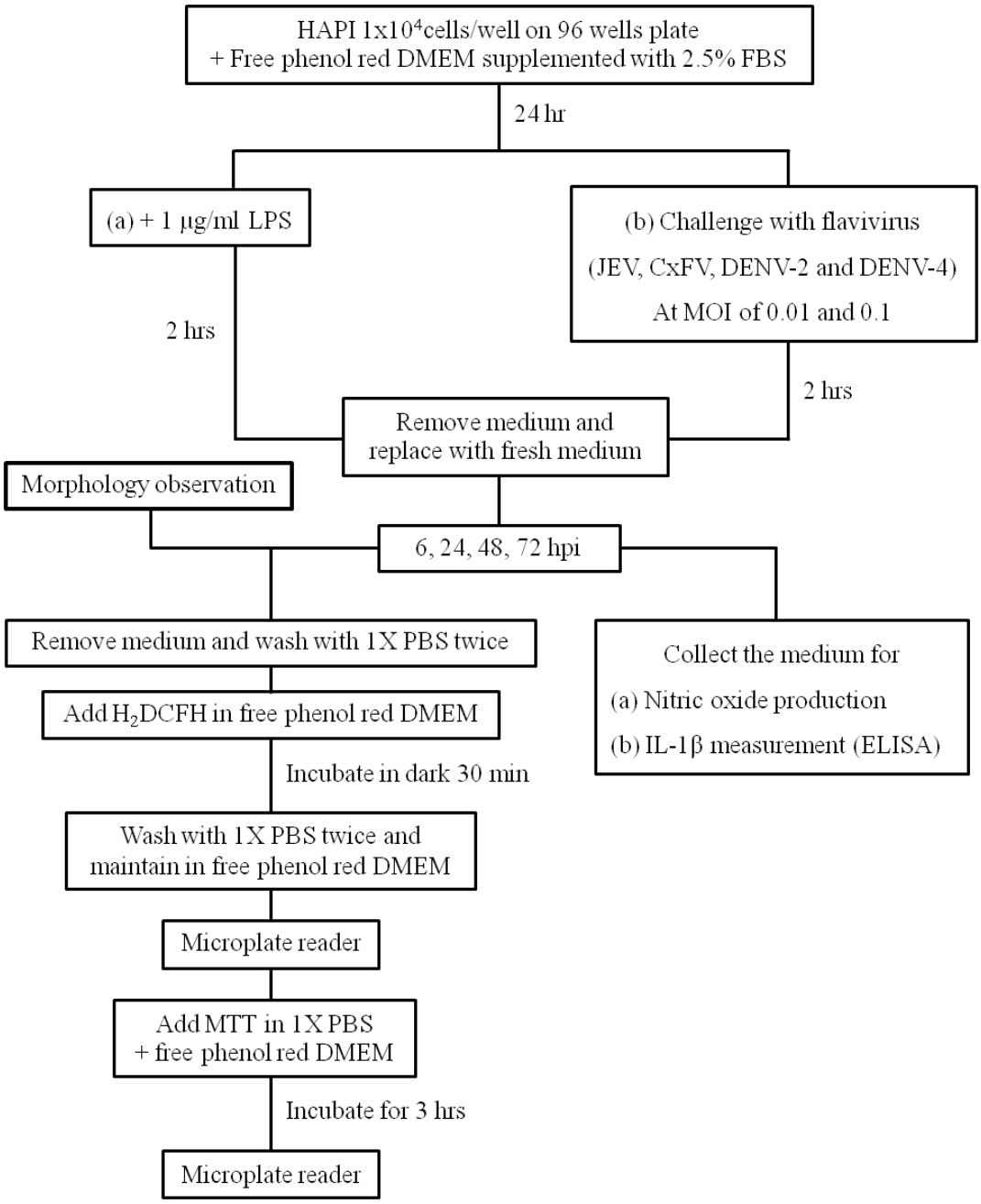

Figure 4.2 Experimental flow chart study the effects of flavivirus induced ROS production in HAPI cells

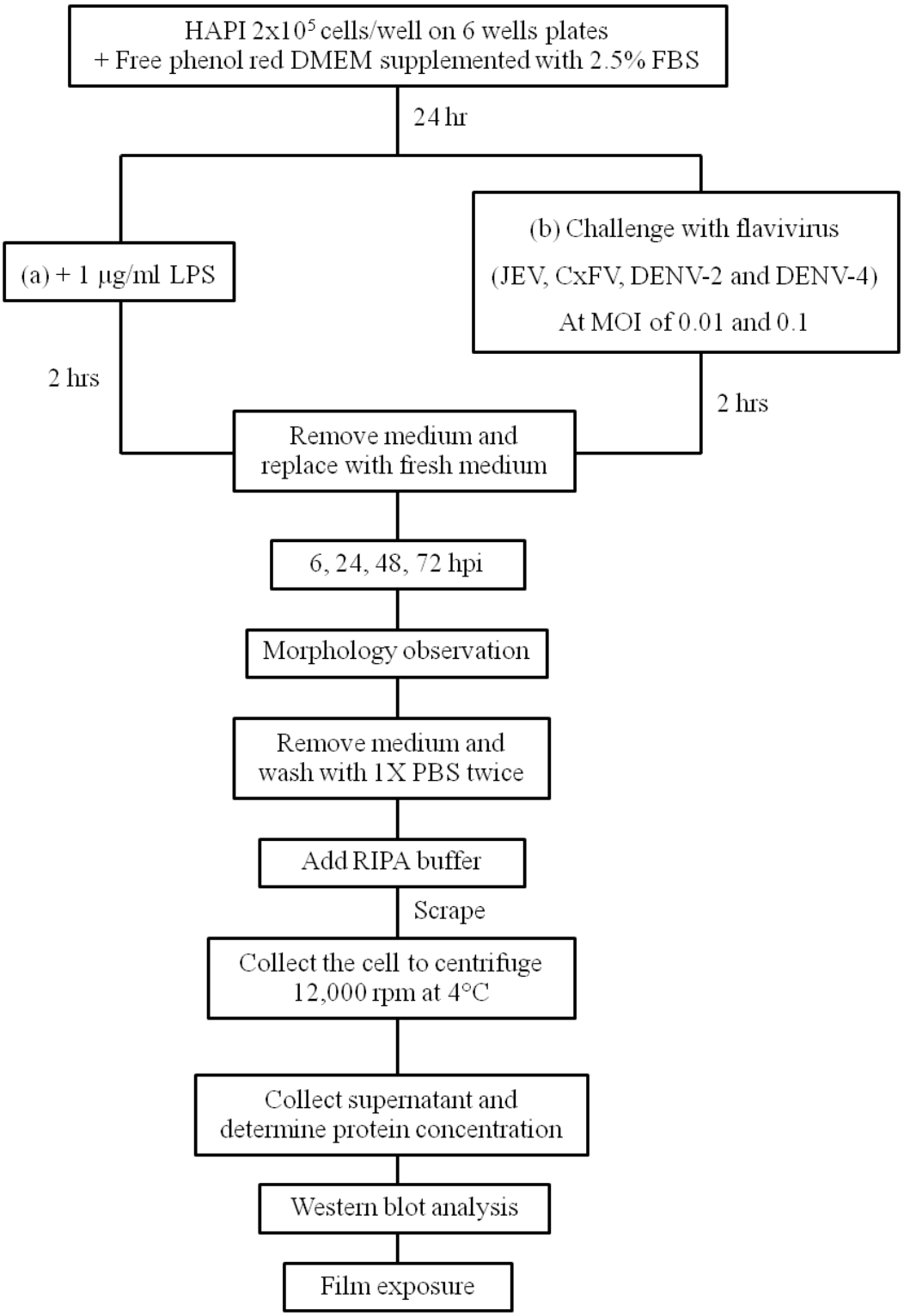

Figure 4.3 Experimental flow chart study the response of HAPI cells followed the flavivirus infection