CHAPTER III

MATERIALS AND METHODS

3.1 Source of Materials

Chemicals

30% Acrylamide and bis-acrylamide solution	BIORAD
Ammonium persulfate	BIORAD
Calcium chloride dehydrate	SIGMA
Coomassie Brilliant Blue R-250	BIO BASIC Inc.
D-Glucose	SIGMA
DAPI (4'-6-Diamino-2-phenylindole)	Invitrogen
di-Sodium hydrogenphosphate anhydrous	Scharlau
Glycine	BIORAD
Lactalbumin hydrolysate	Fluka
Lead acetate	Electron microscopy science
Lead nitrate	Electron microscopy science
Magnesium chloride hexahydrate	SIGMA
Magnesium sulfate heptahydrate	Amersco
3-Methyladenine	SIGMA
Phosphate Buffered Saline (PBS)	SIGMA
Potassium chloride	SIGMA
Potassium dichromate	MERCK
Potassium dihydrogenphosphate	Scharlau
Seakem LE agarose	CamBrex
Sodium citrate	Electron microscopy science
Sodium Dodecyl Sulfate (SDS)	SIGMA
Sucrose	Electron microscopy science
Tetramethylenediamine(TEMED)	BIORAD

Toluidine blue	Electron microscopy science
Trichloroacetic acid (TCA)	MERCK
Tris-base	Vivantis biochemical
Triton X-100	SIGMA
Trypan Blue dye	SIGMA
Tween- 20	SIGMA
Yeast extract	MERCK

Other general chemicals and solvents used but not listed here were purchased from a variety of suppliers. All chemicals used were analytical grade.

Cell culture reagents

Dulbecco's Modified Eagle's Medium (DMEM)	HyClone
DMSO (Dimethyl sulfoxide)	SIGMA
α-modified Minimal Essential Medium (MEMα)	HyClone
Fetal Bovine Serum (FBS)	HyClone
Penicillin/Streptomycin solution	HyClone
HEPES, Free acid	HyClone
L-Glutamine	HyClone
Sodium pyruvate powder	SIGMA
0.25% Trypsin EDTA	Gibco

Cell lines

CHME-5: human embryonic fetal microglial cell line

LLC-MK2: kidney cell line from *Macaca mulatta* (Rhesus monkey)

C6/36: whole hatch larva of mosquito cell line from *Aedes albopictus*

Japanese encephalitis virus

JEV strain Beijing-1 (BJ-1) genotype III

(accession No. L48961)

Source: Human from China

Antibodies

Antibody diluents	Dako
Pan-specific anti-flavivirus monoclonal E protein antibody (A kind gift from Dr. Duncan R Smith, Mahidol University, Thailand)	
Goat anti-mouse IgG-FITC	Dako
Goat anti-rabbit IgG-horseradish-peroxidase	SIGMA
Goat anti-mouse IgG-horseradish-peroxidase	SIGMA
Rabbit polyclonal anti-MAP-LC3	SIGMA
Swine anti-rabbit IgG-TRITC	Dako

Miscellaneous

Pierce® ECL Western Blotting Substrate	Thermo fisher scientific
Pierce® BCA Protein Assay Kit	Thermo fisher scientific
Prestained Protein marker	Fermentas
PVDF (Polyvinylidene Fluoride) membrane	GE Healthcare
Shandon consul-mount	Thermo fisher scientific
Prolong Gold antifade reagent	Invitrogen

Culture media

CHME-5: Dulbecco's Modified Eagle's Medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 1%HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin

C6/36: α-modified Minimal Essential Medium (MEMα), 10% FBS, 1% L-glutamine, 1% HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin

LLC-MK2: Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, 1% HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin

3.2 Cell culture

The *Aedes albopictus* derived *C6/36* cell line was grown in α-modified Minimal Essential Medium (MEMα) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured at 28°C.

Human microglial CHME-5 and monkey kidney LLC-MK2 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplement with 10% FBS, 1% HEPES 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured in humidified atmosphere (5% CO_2/95% air) at 37°C.

3.3 Determination of cell viability

Attached cells were trypsinized and centrifuged at 1,500 rpm for 5 min at room temperature. Then, the pellet was resuspended in appropriate volume of media and pooled with the detached cells. Aliquots of 20 µl of cell suspension was mixed with 50 µl of 0.4% tryptan blue dye and 30 µl of PBS which calculated as a ratio of 1:5 dilution before counting by hemocytometer. The cells mixture was briefly vortexed and incubated for 5 min at room temperature. Aliquots of 10 µl mixture were loaded into hemocytometer for cell counting under light microscope. The live cells can be seen as unstained cells while the dead cells were identified with blue color staining. Counting cells under light microscope in four 1 x 1 mm squares of one chamber and then determined the average cell number of cells per square to calculate as the cell number/ml.

3.4 JEV propagation in C6/36 cells

The Japanese encephalitis virus (JEV) was propagated in C6/36 cells.C6/36 cells were grown in 75-cm^2 tissue culture flasks at 28°c until 80% confluence. The culture medium was discarded and washed 3 times with PBS. Subsequently, 3 ml of MEMα without FBS containing JEV strain Beijing-1 at an MOI of 1 was added. The process of viral absorption was taken for 2 hr at 28°C. The cells were supplemented with fresh culture medium and further incubated for 5 days. Aliquots of the culture medium will be stored at -80°C until use. Virus titers were determined by standard plaque assay.

3.5 Virus titration by standard plaque assay

After propagating JEV in C6/36 cell, JEV was titrated by plaque assay. The monkey kidney LLC-MK2 cells were cultured in 6-well plates for 2 days before time. Cells were washed with PBS and inoculated with 12-fold dilution of JEV in BA-1 viral diluents. Viral absorption was allowed to proceed for 120 min at 37°C with constant agitation. The JEV-infected monolayer cells were overlaid with 2x nutrient mixed with 1.6% seakem LE agarose and incubated at 37°C for 7 days. The plaques were visioned by fixing cells with the 3.7% formaldehyde for 1 hr

before crystal violet staining. The plaque formation on the monolayer of LLC-MK2 cells were counted and calculated as a JEV viral titers.

3.6 Viral infection of human microglial cells

Human microglial CHME-5 cell line was grown in 75-cm^2 tissue culture flasks. When the cells reach density of $3x10^7$cells/flask, the culture medium was discarded and washed 3 times with PBS. Subsequently, 3 ml of DMEM containing JEV at MOI 10 and 100 was added. After viruses were absorbed into the cells for 2 hr at 37°C with constant agitation, the infected cells were washed 3 times with PBS. After washing, fresh media was added and cells were incubated under standard condition.

3.7 Growth curve analysis of JEV-infected CHME-5 cells

CHME-5 cells were plated in 6-well plates at $7.5x10^4$ cell/well overnight. Then, the culture medium was discarded and replaced with 0.2 ml of DMEM containing JEV at MOI 10 and 100 or DMEM without FBS for mock infection. After viruses were absorbed into the cells for 2 hr at 37°C, the infected cells were washed 3 times with PBS before adding fresh media. Cells were incubated under standard condition for 5 days. Live and dead cell number determined by trypan blue dye exclusion assay were examined at 24 hr interval for 5 consecutive days using a hemocytometer.

3.8 Determination of the percentage of JEV infectivity

Approximately $1x10^5$ CHME-5 cells were cultured onto coverslips for 24 hr under standard condition. Later on, cells were either mock-infected or infected with JEV for 2 hr at an MOI of 10 and 100 and, then, incubated under standard condition for 12, 24 and 48 hr as appropriate. After that, cells were subsequently fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. Cells were incubated with 5% normal horse serum for 30 min to block non-specific binding before incubating with a pan specific anti-Flavivirus antibody produced by hybridoma cell line HB-112 (58) (dilution 1:25) for 2 hr at 37°C followed with goat anti-mouse IgG-FITC secondary antibody for 1 hr at room temperature. Nuclei were counterstained with DAPI for 5 min. The coverslips were then mounted onto glass slides using Prolong Gold antifade reagent. The fluorescent signals were investigated under fluorescence microscopy (Axio observer Z-1, Carl Zeiss, Germany). For

light microscope detection, goat anti-mouse IgG-horse radish-peroxidase was used as a secondary antibody. The numbers of positive cells were counted for determining of percent infectivity (20 fields per slide).

3.9 Determination of autophagic cell death

3.9.1 Transmission Electron Microscope

Mock-infected and JEV-infected CHME-5 cells were scraped and centrifuged at 1,500 rpm for 5 min. Then, supernatant was removed. The cell pellets were immediately immersed in 3% glutaraldehyde for 1hr and post-fixed with 1% osmium tetra oxide. The pellets were dehydrated through graded series of ethanol from 50% to 100%. They were passed through two changes of propylene oxide, before being embedded in plastic media. After polymerization at 60°C for 72 hr semi-thin and ultrathin sections were cut by using ultramicrotome. The semi-thin sections (0.5 µm thick) were stained with toluidine blue, in order to select suitable sections for electron microscopy. The ultrathin sections (70-90 nm thick) were stained with uranyl acetate and lead citrate and were examined under the transmission electron microscope.

3.9.2 Western blotting

3.9.2.1 Protein extraction

The confluent 25-cm2 tissue culture flasks of mock-infected or JEV-infected CHME-5 cells were used in the preparation of protein extracts for SDS-PAGE. Before collecting the cell pellets, culture medium was discarded and cells were washed once with PBS. Then, cells were scraped in cold DMEM and pelleted by centrifugation at 1,500 rpm for 5 min. The cell pellet was lysed in RIPA buffer (50mMTris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM, 1% sodium deoxycholate, 1% SDS,1% Tritonx-100, 1X Cocktail Protease inhibitor). The cell pellets were sonicated and incubated on ice for 30 min before centrifugation at 12,000 xg for 15 min. The concentration of protein was quantified by Pierce® BCA Protein Assay Kit.

3.9.2.2 SDS-PAGE

40 µg of total protein from mock-infected or JEV-infected CHME-5 cells was separated by a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100V for 2 hr.

3.9.2.3 Western Blotting

The proteins were electrotransferred to PVDF membrane at 0.35 mA for 90 min using Mini Trans-Blot® Electrophoresis Transfer Cell. Transferred membranes were incubated with 5% skimmed milk in 0.1% TBS-tween (TBST) for 1hr at room temperature. Then, membranes were incubated with1:1000 dilution of a rabbit polyclonal anti-MAP-LC3 antibody or a 1:4000 dilution of a mouse monoclonal antibody against beta-actin in 5% skimmed milk in TBST overnight at 4 °C. Later on, the membrane was shaken for 1hr at room temperature and washing 3 times with TBST and TBS. Then, the blot was incubated with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody at a dilution of 1:10000 or HRP- conjugated rabbit anti-mouse IgG at a dilution of 1:10000 in 5% skimmed milk in TBST for 1 hr at room temperature. The signals were developed by using the Pierce® ECL Western Blotting Substrate. Band intensity volume was analyzed by Quantity One software (Bio-Rad Laboratories Inc.,).

3.9.3 Detection of autophagic marker by indirect immunofluorescence

Approximately $1x10^5$ CHME-5 cells were cultured onto glass coverslips overnight under standard condition. When the cells reached an appropriate density, the culture medium was discarded and washed 3 times with PBS. Later on, cells were either mock-infected or directly infected for 2 hr with JEV at an MOI of 100. Then, cells were washed 3 times with PBS and fresh media was subsequently added. Cells were incubated under standard condition for 48 hr. After that, cells were fixed with 4% paraformaldehyde for 20 min. Cells were washed 2 times with PBS and incubated with rabbit polyclonal anti-MAP-LC3 antibody (dilution 1:200) or a pan specific anti-Flavivirus antibody produced by hybridoma cell line HB-112 (61) (dilution 1:25) as appropriate incubate for2 hr at 37°C. Subsequently, TRITC-conjugated swine anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG was further incubated for 1 hr at room temperature. Nuclei were later counterstained with DAPI. The positive numbers of punctuate LC3 staining were counted and investigated under fluorescence microscopy (20 fields per slide).

3.9.4 Chemical treatment and JEV infection in CHME-5 cells

Approximately $2x10^6$ CHME-5 cells were grown in 75-cm^2 tissue culture flasks for 24 hr under standard condition. The culture medium was discarded and replaced with complete culture medium containing 10mM 3-methyladenine (3-MA) for 3 hr before challenged with JEV at an MOI of 100 for 2 hr. Then, cells were washed once with PBS and then treated with acid glycine buffer (pH 3.0) for 1 min to wash out any un-internalized viruses. Following acid glycine treatment, cells were washed again with PBS. After washing, fresh medium was added and cells were incubated for48 hr under standard condition. The culture supernatants were collected at 24 and 48 hr. and standard plaque assay was performed for virion production.