CHAPTER 2

MATERIALS AND METHODS

2.1 Drug and Chemicals

Thiopental was purchased from Roztoky, Czech Republic. Sodium hydroxide was obtained from Merck (Darmstast, Germany) and all other chemicals were from Sigma Chemical Co. (St. Louis, USA). All chemicals used in the present study were of analytical grade.

2.2 Animal Preparation

Male Wistar rats weighing 300-350 g were obtained from the National Laboratory Animal Center, Nakorn Pathom, Thailand. They were housed individually under standard conditions of temperature 24 ± 1°C and 55 ± 5% relative humidity with regular 12 h light/dark cycle. Food and water were given ad libitum throughout the study. The animals were allowed one week to acclimatize before starting the experiment. All procedures were conducted in conformity with the guidance for the use of animals by the National Research Council of Thailand and were approved by the Institutional Animal Ethics Committee of Chiang Mai University, Chiang Mai, Thailand.

2.3 Isolation of Kidney Mitochondria

Rat was anesthetized by an intraperitoneal injection of thiopental (80 mg/kg body wt) and a systemic perfusion was performed with ice-cold physiological saline solution before excision of the kidneys. Mitochondrial fraction was prepared according to the method described by Piotrkowski et al (56) with slight modification (Figure 2-1).

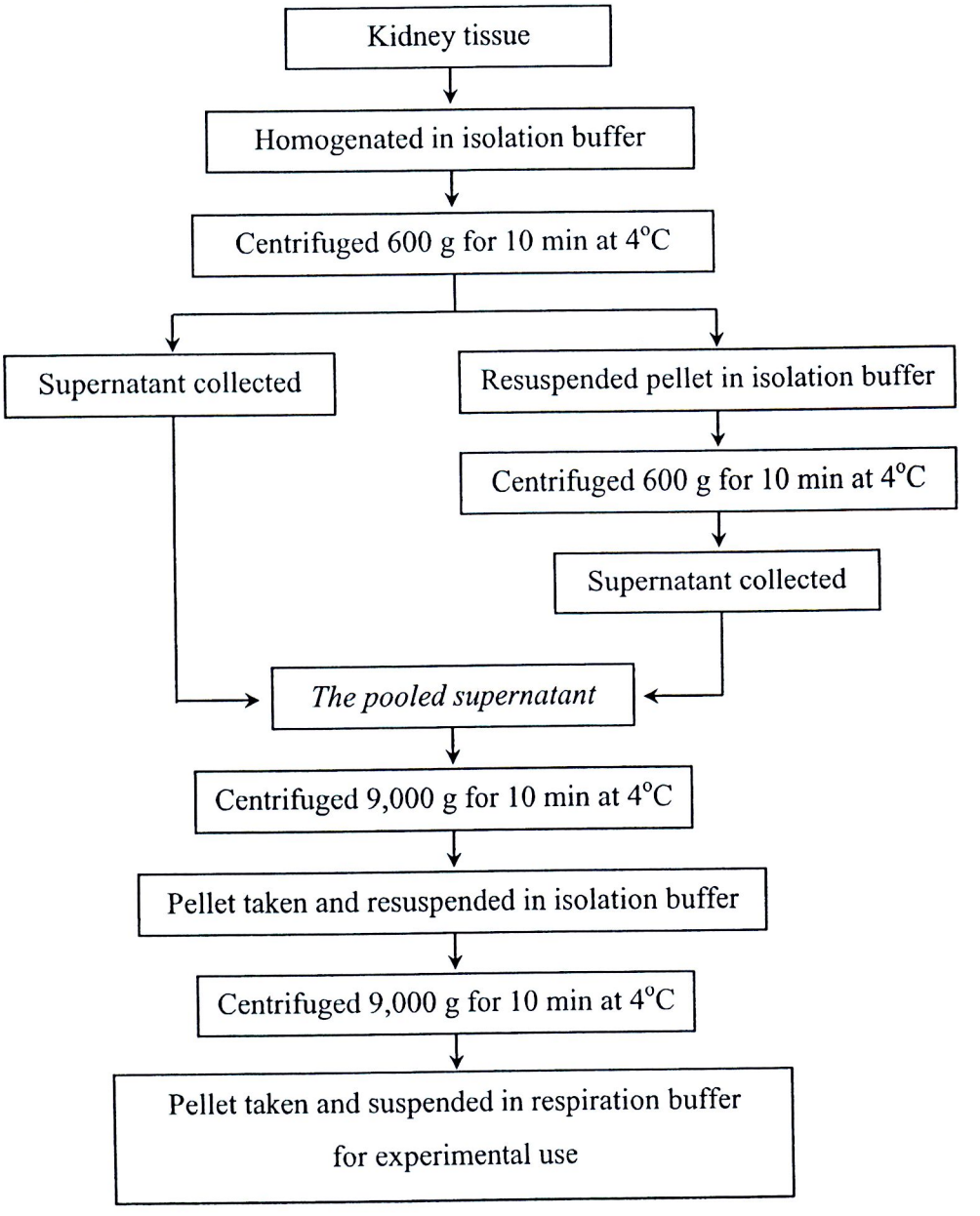

Figure 2-1 Diagrammatic showing the method for isolation of mitochondria

The kidneys were cut into small pieces and homogenized using a Potter Elvehjem homogenizer (Wheaton Science, Millville, NJ, USA), 3 cycles of 3 min with an interval of 1 min between the cycles, in 10 ml of an isolation buffer containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4 at 4°C. The homogenate was centrifuged at 600 g for 10 min at 4°C to eliminate nuclei and cellular debris. The supernatant containing mitochondria was collected while the pellet was resuspended in the same buffer and centrifuged again at 600 g for 10 min at 4°C. The supernatant obtaining from this centrifugation was pooled with the previous collection, and then it was further centrifuged at 9,000 g for 10 min at 4°C. The ensuing pellet was washed once and resuspended in isolation buffer and centrifuged at 9,000 g for 10 min at 4°C. The final mitochondrial pellet was suspended in 1 ml of ice-cold respiration buffer containing 250 mM sucrose, 5 mM KH_2PO_4, 10 mM Tris-HCl, 2 mg/ml BSA, pH 7.2 (57). The protein content of mitochondrial suspension was quantified and mitochondria were used for all experiments within 4 hours of isolation. Each experiment was done in triplicate.

2.4 Experimental Protocols

2.4.1 Protocol 1: Effects of cadmium on kidney mitochondrial function

This protocol not only examined the effects of cadmium on mitochondrial function but also identified the most appropriate dose of cadmium for using in the subsequent protocols. The kidney mitochondrial proteins (0.4 mg/ml) were incubated in respiration buffer containing 250 mM sucrose, 5 mM KH_2PO_4, 10 mM Tris-HCl, 2 mg/ml BSA (pH 7.2) in the presence of cadmium chloride ($CdCl_2$) at various concentrations (10-40 μM) for 5 min at 4°C. The untreated mitochondria were used

as control. The alterations in mitochondrial function were evaluated by detection of mitochondrial swelling, mitochondrial reactive oxygen species (ROS), and mitochondrial membrane potential change. The detail of study protocol was shown in Table 2-1.

Table 2-1 Experimental design for study protocol 1

Group	Description	Treatment
M (n = 6-8)	Control mitochondria	None
MCd 10 (n = 6-8)	Cd 10-treated mitochondria	Mitochondria incubated with 10 μM $CdCl_2$
MCd 20 (n = 6-8)	Cd 20-treated mitochondria	Mitochondria incubated with 20 μM $CdCl_2$
MCd 30 (n = 6-8)	Cd 30-treated mitochondria	Mitochondria incubated with 30 μM $CdCl_2$
MCd 40 (n = 6-8)	Cd 40-treated mitochondria	Mitochondria incubated with 40 μM $CdCl_2$

2.4.2 Protocol 2: Effects of CAPE on kidney mitochondrial function following cadmium exposure

This protocol was undertaken to determine the potential benefit of CAPE on cadmium-induced mitochondrial dysfunction. The kidney mitochondria (0.4 mg/ml) were incubated with various concentrations of CAPE (0.1, 1, and 10 μM) in the presence or absence of $CdCl_2$. CAPE was added 5 min before $CdCl_2$ treatment. The dose of $CdCl_2$ used in this protocol was selected from the most appropriate dose

achieved from protocol 1, which based on its consistency to induce moderate degree of mitochondrial dysfunction. The untreated mitochondria as well as cadmium-treated mitochondria were used as references. Mitochondrial swelling, mitochondrial ROS, and mitochondrial membrane potential change were evaluated. The detail of study protocol was summarized in Table 2-2.

Table 2-2 Experimental design for study protocol 2

Group	Description	Treatment
M (n = 6-8)	Control mitochondria	None
M+CAPE0.1 (n = 6-8)	CAPE 0.1-treated mitochondria	Mitochondria incubated with 0.1 μM CAPE
M+CAPE1 (n = 6-8)	CAPE 1-treated mitochondria	Mitochondria incubated with 1 μM CAPE
M+CAPE10 (n = 6-8)	CAPE 10-treated mitochondria	Mitochondria incubated with 10 μM CAPE
MCd (n = 6-8)	Cd-treated mitochondria	Mitochondria incubated with $CdCl_2$
MCd+CAPE0.1 (n = 6-8)	Cd-treated mitochondria in the presence of CAPE 0.1 μM	Mitochondria pretreated with 0.1 μM CAPE for 5 min prior to the addition of $CdCl_2$
MCd+CAPE 1 (n = 6-8)	Cd-treated mitochondria in the presence of CAPE 1 μM	Mitochondria pretreated with 1 μM CAPE for 5 min prior to the addition of $CdCl_2$
MCd+CAPE10 (n = 6-8)	Cd-treated mitochondria in the presence of CAPE 10 μM	Mitochondria pretreated with 10 μM CAPE for 5 min prior to the addition of $CdCl_2$

2.4.3 Protocol 3 : Effects of cadmium and CAPE on mitochondrial oxidative stress

This protocol was established to clarify whether the effects of cadmium and CAPE were mediated through oxidative stress mechanism. The kidney mitochondria (1 mg/ml) were treated with CAPE or $CdCl_2$ or both. The most effective dose of CAPE obtained from protocol 2 was only studied, while the same dose of $CdCl_2$ as in protocol 2 was utilized. Malondialdehyde and reduced glutathione were analyzed. Table 2-3 displayed the detail of this study protocol.

Table 2-3 Experimental design for study protocol 3

Group	Description	Treatment
M (n = 6-8)	Control mitochondria	None
M+CAPE (n = 6-8)	CAPE-treated mitochondria	Mitochondria incubated with CAPE
MCd (n = 6-8)	Cd-treated mitochondria	Mitochondria incubated with $CdCl_2$
MCd+CAPE (n = 6-8)	Cd-treated mitochondria in the presence of CAPE	Mitochondria pretreated with CAPE for 5 min prior to the addition of $CdCl_2$

2.4.4 Protocol 4 : Effects of cadmium and CAPE on mitochondrial structure

This protocol was carried out to examine the alterations in mitochondrial structure following cadmium and CAPE administration. The same experimental design as in protocol 3 was performed and the electron microscopic examination was used to demonstrate the change in mitochondrial structure.

2.5 Determination of Mitochondrial Protein

The protein concentration of mitochondria was determined by bicinchoninic acid assay (58). Briefly, the standard working reagent was prepared from 100 ml of reagent A (0.1 g sodium bicinchoninate, 0.2 g $Na_2CO_3.H_20$, 0.16 g sodium tartrate (dihydrate), 0.4 g NaOH, 0.95 g $NaHCO_3$, pH 11.25) and 2 ml of reagent B (0.4 g $CuSO_4.5H_2O$ in 10 ml of water). The reaction medium consisted of 50 µl aliquot of the kidney mitochondrial suspension and 1 ml of the standard working reagent. The mixture was incubated at 60°C for 30 min and, after cooling at room temperature, the absorbance was read using a spectrophotometer (UV 1700, Shimadzu Corporation, Kyoto, Japan) at 562 nm. The protein content of mitochondria was quantified from a standard curve of bovine serum albumin (BSA) (59).

2.6 Determination of Mitochondrial Swelling

A light-scattering technique was used to detect mitochondrial swelling (4). This technique monitors the scatter of light resulting from the swelling of a mitochondrial suspension. The amount of light scattered inversely correlates with mitochondrial size such that an increase in mitochondrial volume results in a decrease in light scattering and, hence, absorbance (Figure 2-2).

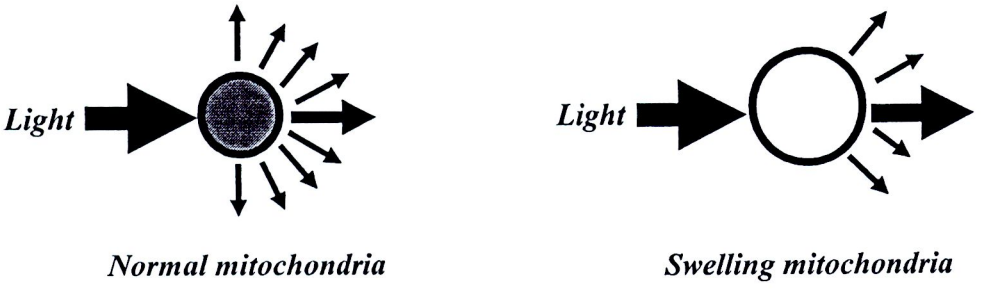

Figure 2-2 Simple diagram showing a light-scattering technique

After exposure of mitochondria to various experimental conditions, mitochondrial swelling was assessed by recording the change in absorbance at 540 nm over 10 min (59, 60). Kinetic measurements were carried out every 30 sec at 25°C using a microplate reader (Synergy™ HT, BIO-TEK® Instruments, Inc., Vermont, USA).

2.7 Determination of Mitochondrial ROS

Mitochondrial ROS production was assayed using a cell-permeable fluorogenic probe 2',7'-dichlorofluorescein diacetate (DCFDA). DCFDA can diffuse into cells and be deacetylated by cellular esterases into the membrane impermeable non-fluorescent dichlorofluorescein (DCFH) (Figure 2-3). In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF). Thus, the fluorescent intensity is proportional to the amount of ROS which are produced by the mitochondria (59, 61).

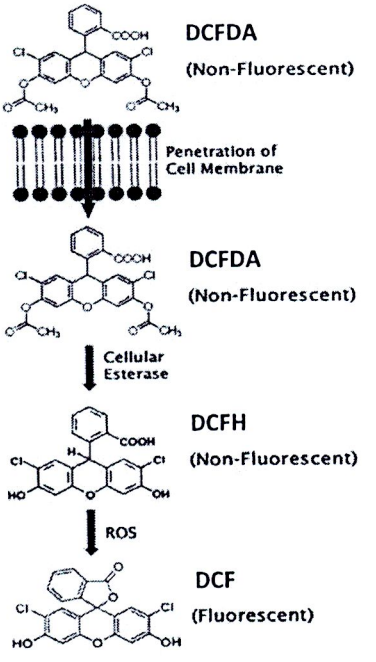

Figure 2-3 Principle of mitochondrial ROS assay

Mitochondria (0.4 mg/ml) were incubated at 25°C with 2 μM DCFDA for 60 min. The fluorescence emission from DCF was determined by a fluorescence microplate reader (Synergy™ HT, BIO-TEK® Instruments, Inc., Vermont, USA) with excitation and emission spectra set at 485 nm (bandwidth 10 nm) and 530 nm (bandwidth 5 nm), respectively. To minimize variation between experiments, the fluorescence intensity was normalized in relation to that obtained from its respective control mitochondria and expressed as an arbitrary unit.

2.8 Determination of Mitochondrial Membrane Potential

A lipophilic cationic fluorescence dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazocarbocyanine iodide (JC-1) was used to assess mitochondrial membrane potential change. JC-1 can selectively enter into mitochondria and reversibly change color as the membrane potential changes. JC-1 primarily forms red fluorescent J-aggregates in physiologically polarized cells, while it generally accumulates in the mitochondria as green fluorescent monomers in depolarized cells (62, 63).

Mitochondria (0.4 mg/ml) were stained with 310 nM JC-1 and incubated at 37°C for 30 min in the dark. A fluorescence microplate reader (Synergy™ HT, BIO-TEK® Instruments, Inc., Vermont, USA) was used to detect the J-aggregates form of JC-1 using an excitation of 485 nm and an emission at 590 nm as well as the monomeric form of JC-1 at excitation and emission wavelengths of 485 and 530 nm, respectively (59, 64). Changes in mitochondrial membrane potential reflected by different forms of JC-1 were quantified, normalized and expressed as a relative value

to the control mitochondria. A decrease in the red/green fluorescence intensity ratio indicates mitochondrial membrane depolarization.

2.9 Determination of Malondialdehyde

Malondialdehyde (MDA), an index of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substances (TBARS) according to the method previously described by Ohkawa et al (65). The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA.

Briefly, the assay mixture consisted of mitochondrial suspension, 10% trichloroacetic acid (TCA), 0.12 M TBA, and 0.2% w/v butylated hydroxytoluene (BHT). This amount of BHT completely prevents the formation of any nonspecific TBARS. The reaction mixture was heated at 90-95°C for 30 min. After cooling, the precipitate was removed by centrifugation at 3,000 g for 10 min at 4°C. The absorbance of the supernatant was read by a microplate reader (iMark, Bio-RAD Inc., CA, USA) at 532 nm. The TBARS level was quantified from a standard curve of 1,1,3,3-tetramethoxypropane and expressed as μM/mg mitochondrial protein.

2.10 Determination of Reduced Glutathione

Reduced glutathione (GSH) was estimated by the method of Sedlak and Lindsay (66). The mitochondrial preparation was precipitated with 10% trichloroacetic acid, centrifuged at 1,500 g for 5 min to obtain supernatant which was then mixed with Ellman's reagent (DTNB in Tris-HCl buffer, pH 8.9). DTNB was reduced by free sulhydryl (–SH) groups of reduced glutathione to form 5-mercapto-2-

nitrobenzoate and its absorbance were measured at 412 nm within 5 min. Results were calculated from a standard curve of reduced glutathione and expressed as μM/mg mitochondrial protein.

2.11 Electron Microscopic Studies

The electron microscopic technique was performed as previously described (67). Briefly, mitochondria were fixed by mixing equal amounts (v/v) of the mitochondrial pellets with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4°C overnight. After rinsing in cacodylate buffer, mitochondrial pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature, dehydrated in a graded series of ethanol, rinsed in propylene oxide, and embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on copper grids, stained by floating on a fresh 50% aqueous saturated solution of uranyl acetate for 12 min, rinsed twice with distilled water and stained immediately by lead citrate. The sections were then examined using a transmission electron microscope (JEM-1200 EX II, JEOL, Tokyo, *Japan*).

2.12 Statistical Analysis

All values are presented as means ± SEM. Comparisons of the differences were performed by a one way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test using the SPSS 16.0 Software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.