# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Instruments, equipments, chemicals and reagents

Details of chemicals and reagents are shown in appendix.

## 2.2 Animal Preparation

All animal studies were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) Faculty of medicine, Chiang Mai University. In this study, Male or female or both sexes normal Wistar rats (300 to 350 g) receive standard pelted rat diet and water *ad libitum*. All rats were housed in stainless steel cages at ambient temperature (20-22°C), humidity and controlled lighting (12-hour day/night cycle). Furthermore, the adult rats were sacrificed and the hearts were removed. Isolated cardiac mitochondria from rat hearts were used in all experimental protocols.

**Experimental 1**

| Group | Treatment | n | Time |
|---|---|---|---|
| 1 | Control (vehicle) | 10 | |
| 2 | Hydrogen peroxide (2mM) only | 10 | |
| 3 | G-CSF (50 ng/ml) only | 10 | |
| 4 | G-CSF (200 ng/ml) only | 10 | |
| 5 | G-CSF + $H_2O_2$ (G-CSF 25 ng/ml followed by $H_2O_2$ 2 mM) | 10 | $H_2O_2$ is added 30 min after given G-CSF. |
| 6 | G-CSF + $H_2O_2$ (G-CSF 50 ng/ml followed by $H_2O_2$ 2 mM) | 10 | $H_2O_2$ is added 30 min after given G-CSF |
| 7 | G-CSF + $H_2O_2$ (G-CSF 100 ng/ml followed by $H_2O_2$ 2 mM) | 10 | $H_2O_2$ is added 30 min after given G-CSF |
| 8 | G-CSF + $H_2O_2$ (G-CSF 200 ng/ml followed by $H_2O_2$ 2 mM) | 10 | $H_2O_2$ is added 30 min after given G-CSF |

**Note** : G-CSF doses used in this study were referred from reference[81] in which subapoptotic dose (0.3 μM) of doxorubicin could induce mitochondrial swelling in cardiomyocytes and could be rescued by G-CSF (100 ng/ml). In this thesis, the doses of G-CSF were varied to optimal the most effective dose of G-CSF that can protect cardiac mitochondrial damage under hydrogen peroxide-induced oxidative stress condition in isolated mitochondria. The number of animal in each group is selected based on the previous reports in which 8-10 rats/group was sufficient to indicate statistical significance.[21, 53, 60]

***Preparation of Mitochondria from Rat's Hearts***

Rats were anesthetized by an intraperitoneal injection of thiopental (80 mg/kg), and the hearts were removed and homogenized in ice-cold buffer containing (mmol/l) sucrose 300, TES 5 and EGTA 0.2, pH 7.2 (4°C).[82] The tissue was finely minced and homogenized by the homogenizer. Afterward, the homogenate was centrifuged at 800*g* for 5 min and the supernatant was collected and centrifuged at 8,800 *g* for 5 min. Mitochondrial pellet was resuspended in ice-cold buffer and centrifuged one more time at 8,800 *g* for 5 min. Protein concentration was determined according to the bicinchoninic acid (BCA) assay.[83] Reagent A was the composition of sodium bicinchoninate (0.1 g), $Na_2CO_3.H_2O$ (2.0 g), sodium tartrate (dihydrate) 0.16 g, NaOH (0.4 g), and $NaHCO_3$ (0.95 g), made up to 100 ml. The pH will be adjusted to 11.25 with $NaHCO_3$ or NaOH, if necessary. Reagent B was the composition of $CuSO_4.5H_2O$ (0.4 g) in 10 ml of water and the standard working reagent (SWR) is a mix of 100 vol of reagent A, with 2 vol of reagent B. The solution is apple green in color and is stable at room temperature for 1 wk. SWR (1

ml) was added to mitochondrial protein (50 μl) and incubated at 60°C for 30 min. The sample was cooled to room temperature prior absorbance measurement at wave length 562 nm using a spectrophotometer. A calibration curve was constructed using dilutions of a stock 1 mg/ml solution of bovine serum albumin (BSA).

**2.3 Study Protocols**

***Protocol I: To identify the cardiac mitochondria with electron microscopy***

Electron microscopy was used to identify isolated cardiac mitochondria.[84] Isolated mitochondria was fixed by the composition of the mitochondrial suspensions with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4°C. After rinsing in cacodylate buffer, mitochondrial pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 hr at room temperature, and then dehydrated in a graded series of ethanol. Mitochondria were embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on copper grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope.

***Protocol II: To study the effects of G-CSF on mitochondrial swelling in isolated mitochondria***

All groups of isolated mitochondria were tested for mitochondrial swelling. In this protocol, mitochondrial swelling was assessed by measuring the change in the absorbance of the suspension at 540 nm (*A*540) using a microplate reader.[85] Mitochondria (0.4 mg/ml) was incubated in 1.5 ml respiration buffer (100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM $KH_2PO_4$, pH 7.4 at 37°C) for 1 min.

Mitochondrial swelling was detected when the absorbance of the suspension decreased.

***Protocol III: To study the effects of G-CSF on ROS production in isolated mitochondria***

ROS production in mitochondria was measured using a fluorescent microplate reader with dichlorohydro-fluorescein diacetate (DCFDA).[86] Heart mitochondria (0.4 mg/ml) were incubated C with 2 μM DCFDA at 25° for 20 min. The mitochondrial suspension was gently agitated and incubated at room temperature for measurements. DCFDA was oxidized in the presence of $H_2O_2$ to DCF. Fluorescence was determined at $\lambda_{ex}$ 485 nm (bandwidth 10 nm) and $\lambda_{em}$ 530 nm (bandwidth 5 nm) according to the spectral characteristics of DCF. The ROS formation was expressed as arbitrary units of fluorescence intensity of DCF.

***Protocol IV: To study the effects of G-CSF on mitochondrial membrane potential (ΔΨ) changes in isolated mitochondria***

The changes in mitochondrial membrane potential from all groups of isolated mitochondria were monitored with the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1).[87, 88] JC-1 is a ratiometric dye that is internalized as a monomer dye (green fluorescence, emission wavelength 530 nm) and is concentrated by respiring mitochondria with negative inner membrane potential into J-aggregate dye (red fluorescence, emission wavelength 590 nm). Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The isolated mitochondria (2 mg/ml) were incubated by respiration

buffer with JC-1 (310 nM) at 37 °C for 15 min. The intensity of fluorescence was determined using a fluorescent microplate reader. JC-1 monomer (green) fluorescence was excited at 485 nm and the emission was detected at 530 nm. JC-1 aggregate (red) fluorescence was excited at 485 nm and the emission fluorescence was recorded at 590 nm. Mitochondrial depolarization was indicated by a decrease of fluorescence intensity.

**Experimental study 2**

In this study, isolated mitochondria were randomly assigned into twelve groups to investigated the mechanism of action of G-CSF. Cyclosporine A (CsA, a mitochondrial permeability transition pore (mPTP) inhibitor) and 4'-chlorodiazepam (CDP, an inner membrane anion channel (IMAC) blocker ) were used to clarify whether G-CSF acts on mPTP or IMAC in isolated cardiac mitochondria. CDP is known to inhibit the opening of IMAC, whereas CsA is the mPTP blocker.

| Group | Treatment | n | Time |
|---|---|---|---|
| 1 | Control (vehicle) | 10 | |
| 2 | G-CSF (50 ng/ml) only | 10 | |
| 3 | Cyclosporine A 5 μM only | 10 | |
| 4 | 4'Cl-DZP (CDP) 100 μM only | 10 | |
| 5 | $H_2O_2$ 2 mM only | 10 | |
| 6 | G-CSF + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given G-CSF |
| 7 | CsA + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given CsA |
| 8 | CDP + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given CDP |
| 9 | (G-CSF + CsA) + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given (G-CSF + CsA) |
| 10 | (G-CSF + CDP) + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given (G-CSF + CDP) |
| 11 | (CsA + CDP) + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given (CsA + CDP) |
| 12 | (G-CSF + CsA + CDP) + $H_2O_2$ | 10 | $H_2O_2$ is added 30 min after given (G-CSF + CsA + CDP) |

**Data analysis**

Results of each experimental study were analyzed and translated. The information was compared among groups to indicate whether G-CSF is able to protect cardiac mitochondrial damage and to determine the effective dose of G-CSF that is able to protect cardiac mitochondrial damage. In addition, the mechanism of G-CSF in isolated mitochondria was investigated. All data were presented as means ± SEM and analyzed by one-way ANOVA followed by the Fisher procedure to compare the mean values. A $P$ value $< 0.05$ was considered statistically significant.