CHAPTER II

MATERIALS AND METHODS

Animal Care

The experiments were performed using male Wistar rats, weighting between 220-250 g. The animals were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand. All animals were housed two per cage in air-conditioned room and provided with food and water *ad libitum*. The temperature and lighting were constantly controlled approximately at 24-25 ˚C and 12 hr light-dark cycle, respectively. The animals were acclimatized 1 week before the beginning of the experiment.

Reagents

The trade name of a dicrotophos used in this study is Canoes 33® ($C_8H_{16}NO_5P$, 33% W/V SL dimethyl (E)-1-methyl-2-(dimethylcarbamoyl)-vinylphosphate) which was purchased from Superior Chemical Industry (Thailand) Ltd. The drug was diluted in 0.9% saline for intraperitoneal injection.

Animal Studies

The animals were injected intraperitoneally with daily dose of dicrotophos (3.75 mg/kg BW) for 4 weeks. Animals were tested for nerve conduction velocity, histopathology and AChE activity in red blood cell (RBC), plasma and brain at 24 hrs, 1, 2 or 3 weeks after the last dose. The control rats were injected with saline but nerve conduction velocity, histopathology and AChE activity were determined only at 24 hrs or 3 weeks after the last injection. Schematic presentation of experimental procedure was summarized in Figure 3.

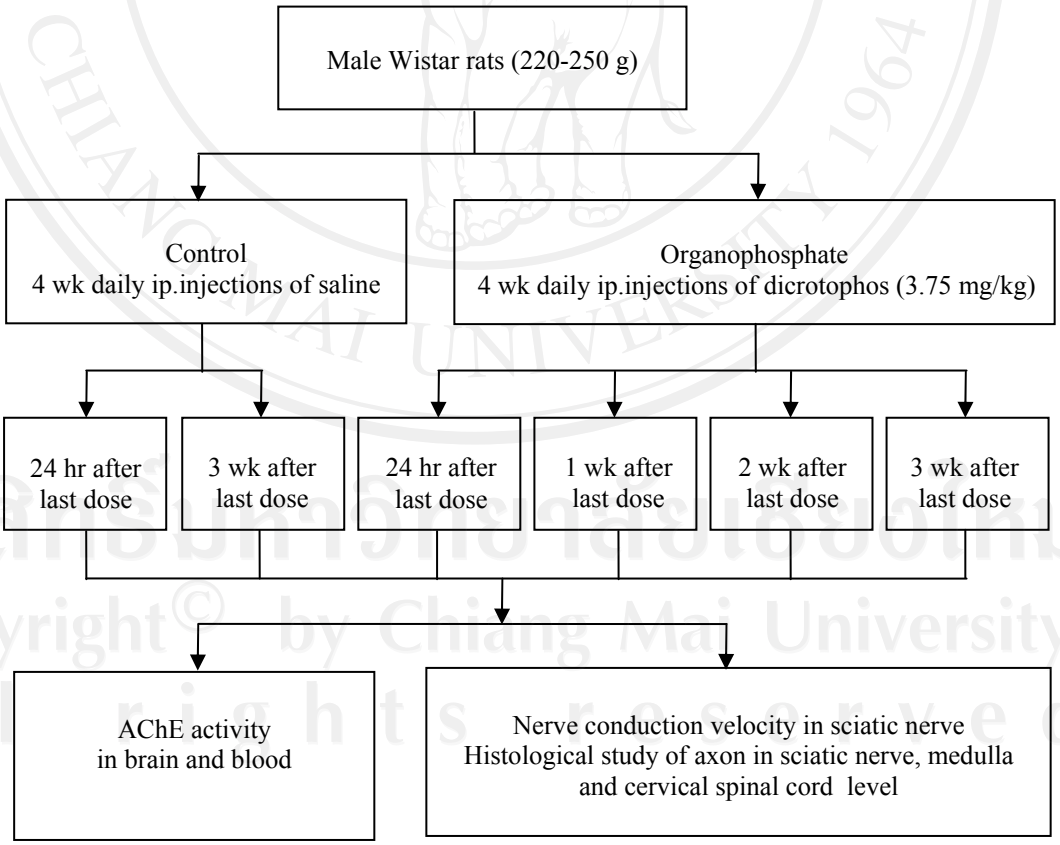


Figure 3 Schematic presentation of experimental protocol.

1. Determination of acetylcholinesterase (AChE) activity

To study the effects of dicrotophos on AChE activity in blood and brain, animals were anesthetized with pentobarbital sodium (50 mg/kg BW) administered intraperitoneally. Then, blood samples were collected and animals were intracardially perfused with 0.9% saline solution (NSS). Whole brains were removed rapidly after careful removal of skulls. Then, blood samples and homogenized brain were used to determine AChE activity.

Preparation of blood samples and brain homogenate.

1. Plasma and red blood cells (RBC)

After blood sample collection, the whole blood samples were centrifuged at 2,500 rpm for 5 min at room temperature. Plasma was separated from RBC and then removed for determination of AChE activity. The remaining blood was transferred to another microtube containing saline and then centrifuged for 5 min at 5,000 rpm. Saline was carefully removed, and 20 µl of packed red cells was transferred into a microtube containing 180 µl of distilled water. The tube was tapped firmly until RBC was lysed. Both plasma and RBC samples were stored until AChE analysis.

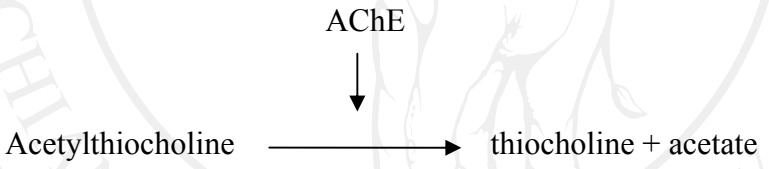
2. Brain homogenate

After intracardial perfusion with NSS, the brain was rapidly removed, weighed and homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4, 4 ˚C) containing 1%

Triton X-100 (~10% W/V). The homogenates were then centrifuged at 15,000 rpm, 4˚C for 15 min. Supernatants were collected and assayed for AChE activity.

Acetylcholinesterase (AChE) activity assay

The AChE activity was assayed following the method described by Ellman *et al* (1961) and modified by Pongrawewongsa and Ruangyuttikarn (1999). The enzyme activity is determined by measuring the yellow color produced when thiocholine reacts with dithiobisnitrobenzoate ion. It is based on coupling reactions as follows.

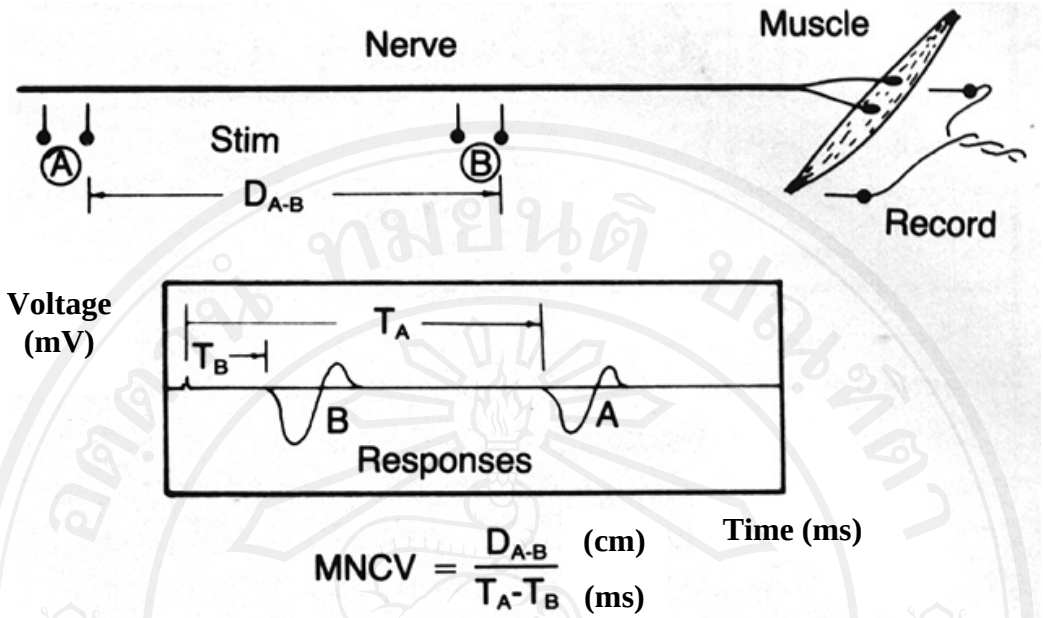


Thiocholine + dithiobisnitrobenzoate ⟶ yellow color

The samples (diluted RBC solution, plasma or brain homogenates, 20 µl) was mixed in 3 ml of 5, 5-dithiobisnitrobenzoic acid solution and 50 µl of acetylthiocholine iodide was added and mixed. The absorbance of the solution was measured at 405 nm using spectrophotometer. AChE activity was then calculated and reported in U/L/g tissue for brain homogenate and U/L for RBC and plasma

2. Determination of nerve conduction velocity

Nerve conduction velocities were determined at 24 hr, 1, 2 or 3 weeks after the completion of dicrotophos treatment, according to the method described by Jamieson *et al* (2005). Briefly, rats were anesthetized with pentobarbital sodium, 50 mg/kg BW, by intraperitoneal injection. The animal was placed in supine position on a plexiglass board. The lower limb of the animal was immobilized using a metal bar attached to the stand. The left sciatic nerve and tibial nerve of the rat were exposed and connective tissue was removed. Stimulating electrodes were placed at the sciatic nerve while recording electrodes were placed at the sole. Stimulation of the sciatic nerve was performed using single stimulus and the resulting electromyogram (EMG) from plantar muscles was recorded, and the latency of the EMG was determined. Stimulation of tibial nerve was performed and the latency of evoked EMG record was determined the same way as for sciatic nerve. Motor nerve conduction velocity (MNCV) was then calculated by dividing the distance between the sciatic and tibial nerve stimulation sites by the latency difference between the onsets of the electromyographic potentials evoked from the two sites. A schematic representation of nerve conduction studies is illustrated in Figure 4.



Ⓐ : sciatic nerve stimulation site

Ⓑ : tibial nerve stimulation site

D_{A-B} : distance between the sciatic and tibial nerve stimulation sites

T_A : the latency of the electromyographic potential evoked from stimulation at sciatic nerve

T_B : the latency of the electromyographic potential evoked from stimulation at tibial nerve

Figure 4 A schematic representation of nerve conduction study (Farnbach, 2007 [online]).

3. Histological study

The animals were trancardially fixed at 24 hr, 1, 2 or 3 weeks after completion of treatment. The physiological saline was initially administered and followed by 5% glutaraldehyde in Sorensen's 0.1 M sodium phosphate buffer (pH 7.4, 4˚C). The medulla, cervical spinal cord and sciatic nerve were dissected and immersed at 4˚C in the glutaraldehyde fixative for additional 24 hr. Tissue samples were then rinsed in 0.1 M phosphate buffer, post-fixed in 2% osmium tetroxide for 2 hr and washed in 0.1 M phosphate buffer. They were thereafter dehydrated in graded alcohols, infiltrated and embedded in plastic. The embedded tissue was cut in 0.9 µm thickness. The 0.9 µm sections were stained with methylene blue and the numbers of myelinated nerve fiber and unmyelinated nerve fiber were counted. The minimal diameter of axon was examined and the thickness of myelin sheath was estimated using a light microscope coupled with an eyepiece micrometer. The plastic embedded tissue was cut again in 0.5 µm thickness at selected area for transmission electron microscopic study. The 0.5 µm sections were stained with lead citrate and urenyl acetate. Electron micrographs of axon in medulla, cervical spinal cord and sciatic nerve were examined.

Statistical Analysis

All values were expressed as means ± SE. The significance of differences among experimental groups was determined using Mann-Whitney *U-test*. A level of $p \leq 0.05$ was considered significant.