CHAPTER III

MATERIALS AND METHODS

This experiment was performed on male Wistar rats, weighing between 200-300 g. All rats were fed ad libitum with standard rat chow (CP, Thailand) and allowed free access of tap water. DOX (Onco-Tain® : containing doxorubicin hydrochloride 2 mg/ml) was obtained from David Bull Laboratories, Australia.

Experimental Protocol

The experiment was separated into 2 sections for studying acute and subacute effects of DOX. Acute effect of DOX was performed in normal rats without DOX injection, while subacute effect of DOX was performed in rats after a course of 2 injections with DOX. On the experimental day, rats of both sections were studied using the atrial muscle preparations.

The Atrial Muscle Preparation

On the day of the experiment, rats were euthanatized by cervical dislocation. After midline thoracotomy, the heart was quickly removed and placed in a dissection dish filled with Krebs-Henseleit solution (KHS : containing in mM: NaCl 118.00, KCl 4.70, $MgSO_4.7H_2O$ 1.20, $CaCl_2$ 2.52, KH_2PO_4 1.18, $NaHCO_3$ 24.88 and glucose 11.08), gassed continuously with 95% O_2 and 5% CO_2 mixture. Fat and connective tissues surrounding the heart was carefully removed and the atria were isolated from the ventricles, followed by dissecting right and left atria. Then, each right and left atria was mounted vertically in a 20-ml double-walled glass organ bath containing KHS. The solution was saturated with a 95% O_2 and 5% CO_2 gas mixture and maintained at 37 °C. The isolated right atrium was sutured at the upper ends with a line of cotton thread, which was hanged on a force transducer (Grass Medical Instruments, MA, USA)

connecting to Physiograph (Grass Model 79 Polygraph®). The lower end of the tissue was fixed with a hook in the organ bath. The isolated left atrium was sutured with a line of cotton thread on the upper end for connecting to a force transducer and transferred to another organ bath. The lower end was hooked with a platinum electrode for electrical stimulation (Grass Model®) at 250 beats per minute (bpm) with square wave pulses 5 milliseconds (msec) at 5 volts (V) in intensity.

Atrial muscle preparations were equilibrated for 45 minutes at a resting tension of 1.0 g. The isometric contraction of atrial muscle preparations were measured by a force transducer and a Physiograph, which was connected to a computer for recording the data throughout the study. The heart rate, right atrial force and left atrial force, used as the parameter, were later analyzed with the recording data stored in the computer.

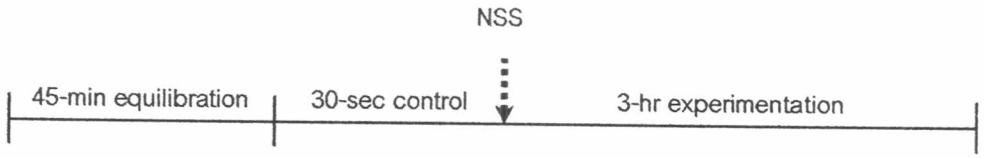
Section 1: Acute Effects of DOX

In 4 experiments of acute effects, 8 rats were used for each experiment. Animals were euthanatized, and the hearts were obtained for examining effects of DOX on rate and force of contraction, post rest contraction, cumulative dose-response curves of isoproterenol and acetylcholine.

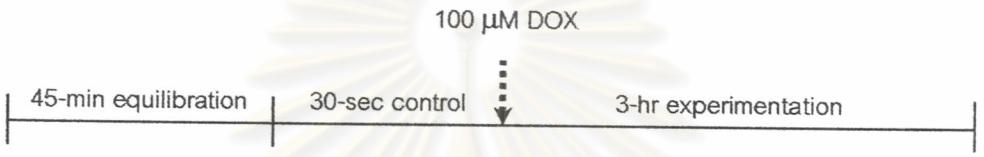
1.1 Effect of DOX on Rate and Force of Contraction

Both right and left atria muscle preparations were used in this experiment. After an equilibration for 45 minutes, the 30-second control period was recorded. DOX was added to the incubation medium. The final concentration of DOX was 100 μM. The same volume of vehicle (normal saline; NSS) was added to another organ bath for control preparation. Rate and force of contraction of right atria and left atrial force were observed every 10 minutes throughout 3 hours of experimentation.

Control group



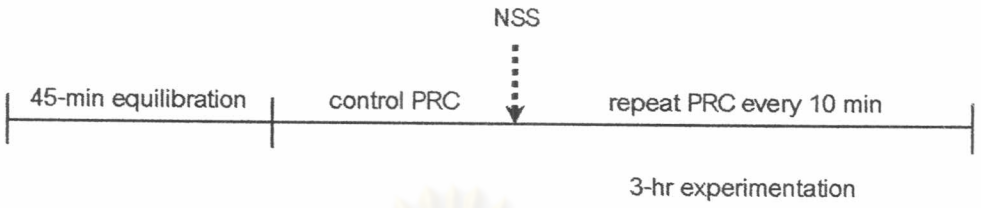
Treatment group



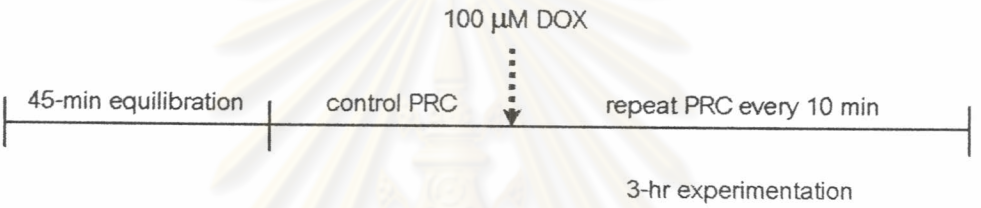
1.2 Effect of DOX on Post-rest Contraction (PRC)

In this experiment, left atrial muscle preparations were stimulated at 60 bpm with the same square wave pulses and intensity. PRC had been suggested to be highly dependent on Ca^{2+} release from the SR. Following 45-minute equilibration, first PRC was performed as a control PRC. After adding 100 µM DOX in the medium, PRCs were examined at 10-minute intervals throughout 3 hours of experiment. The stimulator was turned off for 30 seconds and re-stimulated. The first contraction was observed. The same volume NSS was added to the other organ bath for control preparation. The initial tension after resting period (Ti) was recorded and compared with the steady-state tension (Tss). Ti/Tss was used as an index of releasable Ca^{2+} in SR.

Control group



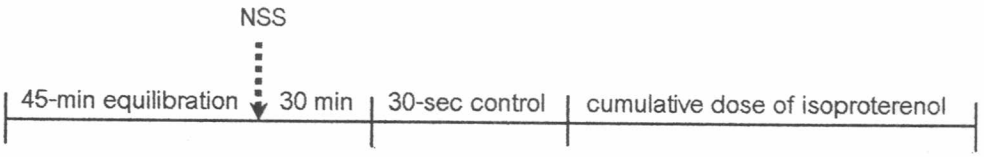
Treament group



1.3 Cumulative Dose-response Curves of Isoproterenol at Doses of 10^{-9} – 10^{-5} M

This experiment was designed to study the direct effect of DOX on beta-adrenergic receptor. After equilibration and control period, either NSS or DOX at a dose of 100 µM was added in the both chambers mounted with isolated right and left atria. After incubation with the drug for 30 minutes, isoproterenol was added to the solution in a cumulative manner of doses ranging from 10^{-9} to 10^{-5} M. Changes of rate and force of contraction were recorded for the cumulative dose-response curve of isoproterenol.

Control group

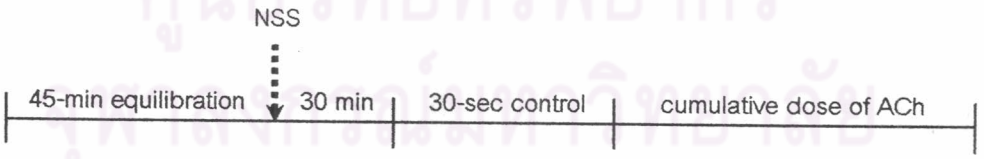


Treament group

1.4 Cumulative Dose-response Curves of Acetylcholine at Doses of $10^{-7} - 10^{-3}$ M

To study the direct effect of 100 μM DOX on muscarinic receptor, acetylcholine was added in doses of cumulative manner in the both chambers mounted with isolated right and left atria after incubation with the drug for 30 minutes. Changes of rate and force of contraction will be observed and compared with those of control preparation.

Control group



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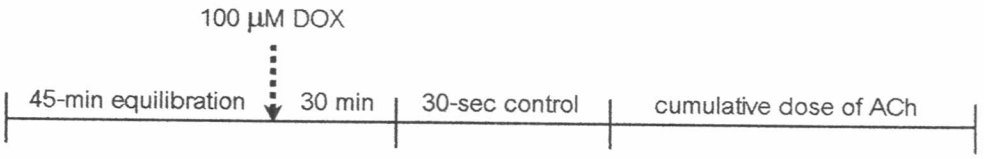

Section 2: Subacute Effects of DOX

In subacute experiments, the rats were randomly divided into 2 groups (control or DOX-treated group). Both groups (8 rats/group) were used in each experiment. After the animals arrived, they were kept in quarantine for 3 days before beginning of the experiments. At the first day of the experiment, the rats were weighed, marked for identification and treated according to the following dose schedule: group 1 received 2.5 mg/kg DOX i.p. and group 2, control group, received vehicle (NSS) i.p. in volume equal to volume of DOX used in group 1. All rats were treated in the same dose again on the seventh day. The total doses of each DOX-treated rat were 5 mg/kg. This selected dose was modified from the dose used in guinea pig (Hagane et al., 1988). The animals were euthanatized on the tenth day.

On day 10, rats of each group were sacrificed by cervical dislocation. Thorax was exposed and heart was removed immediately. The right and left atrial muscle preparations of each group were obtained to evaluate subacute effects of DOX on post rest contraction and cumulative dose-response curve of either isoproterenol or acetylcholine. The protocol of each experiment was similar to the section 1.2, 1.3 and 1.4, respectively without addition of DOX or NSS in the organ bath. Additionally, the ventricles of the same hearts were collected and prepared to determine the myocardial ATPase and CK activities.

Measurements of Myosin ATPase, Actomyosin ATPase and Creatine Kinase Activities

1. Tissue Preparation

Subacute effects of DOX on myosin ATPase, actomyosin ATPase and CK activities were performed on rats treated with DOX and NSS. After hearts were removed immediately from euthanatized rats, the isolated ventricles were washed and minced with scissors in cold solution of 20 mM $NaHCO_3$, followed by homogenization for 2 min in 10 volumes of 20 mM $NaHCO_3$ with a homogenizer. The homogenate was centrifuged

(Sorval® Super T21) at 10,000xg for 30 minutes. Then, the supernatant was separated and used for CK activities determination. Actomyosin in the pellet was extracted with 10 volumes of 0.66 M KCl and 50 mM Tris-maleate at pH 6.8 overnight, followed by dialysation against 0.06 M KCl and 50 mM Tris-maleate at pH 6.8. After dialyzing, the dialysate was centrifuged at 10,000xg for 30 minutes and the pellet was redissolved with 0.66 M KCl and 50 mM Tris-maleate at pH 6.8. The actomyosin solution was kept in freezer at -70 ^{o}C before using for myosin ATPase and actomyosin ATPase determination. The diagram of tissue preparation was shown in Fig. 2.

2. Myosin ATPase, Actomyosin ATPase and Creatine Kinase Activities Determination

2.1 CK activity was determined by the method of Foster et al. (1974)

2.2 Myosin ATPase was determined with actomyosin solution in 0.6 M KCl, 20 mM Tris-maleate at pH 6.8, 1 mM $CaCl_2$ and 1 mM ATP (Watanabe and Kishikawa, 1998).

2.3 Actomyosin ATPase was determined in 0.03 M KCl, 20 mM Tris-maleate at pH 6.8, 1 mM $MgCl_2$, 10 mM $CaCl_2$ and 1 mM ATP. In the absence of Ca^{2+}, 1 mM of ethyleneglycol bis (2-amino ethyl ether) tetraacetic acid (EGTA) was added in another assay mixture (Watanabe and Kishikawa, 1998).

2.4 Inorganic phosphate and protein concentration were measured by the method of Taussky and Shorr (1953) and Lowry et al. (1951), respectively.

Statistical Analysis

The data was presented as mean±S.E. for *n* observations. Significant test of data between control and treatment groups was performed using Student's unpaired *t*-test. One way repeated measures analysis of variance was used for comparison of data within the same group before and after the treatment and the Tukey test was used

for multiple comparisons versus a control. Friedman repeated measures analysis of variance on ranks was used in the nonparametric data. Differences between means of each data were considered statistically significant at $P \leq 0.05$. The program used for this analysis was Sigma-Stat.

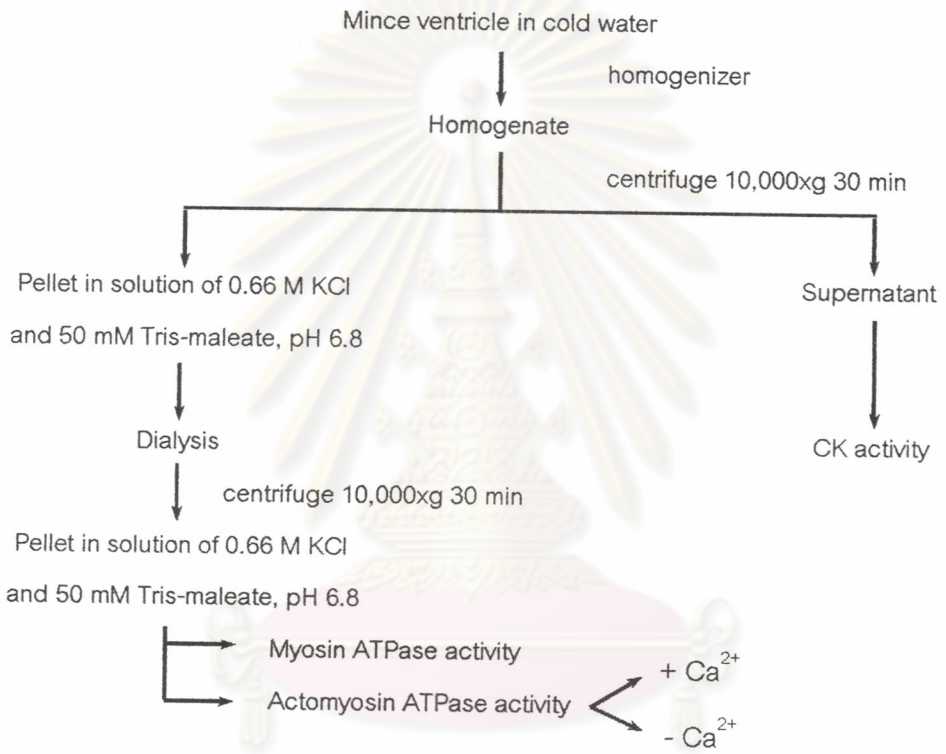

Fig. 2. Diagram of tissue extraction for ATPase and CK activities determination