CHAPTER 4

RESEARCH METHODOLOGY

4.1 Scope of Study, Place and Time of The Research

Scope of the study includes pathobiology and anesthesiology. The study was conducted in Animal Laboratory at Gajah Mada University and the tissue examination was done at Department of Pathology Gajah Mada University in Yogyakarta between March - April 2011.

4.2 Research Design

Study design is experimental study, namely post test only control group design. The samples were randomly divided into 4 intervention groups and 1 group for control.

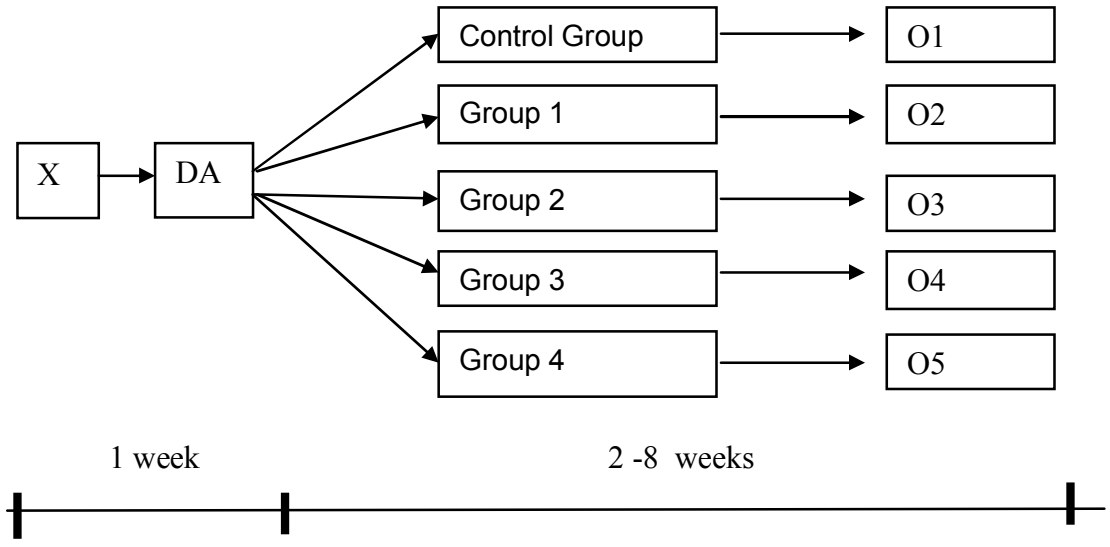

Fig 4. The scheme of the study

Note :

X = Adaptation time

DA = Time Allocation into 4 groups,e.g.

Control Group = group of mice without halothane

Group 1 = exposure to 0,011 mg/weight halothane 3 hours daily for two (2) weeks.[37] The dose is less than the maximum dose for mice (0,03) because the researcher doesn't know how much halothane which is inhaled by medical person.

Group 2 = exposure to 0,011 mg/weight halothane 3 hours daily for four (4) weeks.

Group 3 = exposure to 0,011 mg/weight halothane 3 hours daily for six (6) weeks

Group 4 = exposure to 0,011 mg/weight halothane 3 hours daily for six (6) weeks followed by 2 weeks of free halothane

Every group gets one (1) additional mouse for replacing mice which die during the study period.

O1 = Output from the group control

O2 = Output from the group 1

O3 = Output from the group 2

O4 = Output from the group 3

O5 = Output from the group 5

The dose for halothane are 0.011 mg/weight given 3 hours daily for 2 weeks, 4 week, 6 weeks and 6 weeks follow by 2 week halothane free. This research used fixed dose (0.011 mg/weight)[35] that is optimal dose of halothane on mice. It is based on preliminary experiment that shows this dose did not kill the mice. The previous dose mentioned in the proposal (6 mg/cage), killed the mice directly after introduction of halothane in the cage. Given that situation we reduced the dose gradually until we found the optimal dose. Optimal dose was characterized by activity of mice that was not obviously affected after halothane exposure. Different time of exposure mimics the working time of medical personal in operation room. Exposure of halothane about 3 hours because the averages of operation time by medical personals are 2-6 hours (data from medical person who work in operation room) and researcher will use the median of average operation time. The median of the time is 3 hours.

4.3 Population of The Study

Population of the study are healthy balb/C mice inbred strain of Animal Laboratory at Gajah Mada University. Samples were taken from such mice which were fullfill the following criteria:

4.3.1 Inclusion Criteria

Balb/C mice (Male), in healthy condition and 6–10 weeks old, no infectious disease and the weight are between 29 – 33 grams.

4.3.2 Exclusion Criteria

The Balb/C mice were diarrhea or show behavioral changes (e.g, do not eat/loose of appetite).

4.4 Sample Size

Determine the number of samples based on WHO requirement with a sample of at least 5. Sample in this research is BALB/c Mice (male). The number of the mice were 30 mice, divided into five groups, each of group consist of 6 mice.

4.5 Research Variable

4.5.1 Independent variable

The independent variable was halothane exposure time.

4.5.2 Dependent variable

Dependent variables were changes of liver cells nucleus changes and cytochrome P450 2E1 changes.

4.6 OPERATIONAL DEFINITION

4.6.1 Halothane

Halothane vapor (or Fluothane) is an inhalational general anesthetic agent. The dose for halothane are 0.011 mg/weight given 3 hours daily for 2 weeks, 4 week, 6 weeks, and 6 weeks respectively; and followed by 2 weeks of halothane free. The scale is ordinal.

4.6.2 Liver cells nucleus and cytochrome P450 changes

The liver of each mouse was taken to do histopatological examination by processing the tissues (made into paraffin block) and HE staining. The slides were observed under the light microscope.

Nucleus changes are described as follow: in degeneration stage it will be enlarge, karyorhexis, karyolisis/necrotic of the nucleus.[35,36] Using magnification of 1000 x, the changes of the cells are counted in 10 fields, one time a day, randomize choosing. The total score for each change in one mice was counted and divided into 10. After got scores for all mice, total score in one group was counted by summing number of enlarge, karyorhexis, and karyolisis/necrotic nucleus in six mice. The data is numeric data and the scale is ratio.

We also examined the cytochrome P450 in the liver by immunohistochemistry using cytochrome P450 antibody. The results were based on the colours' intensity of slides, namely "strong (3)", "moderate (2)", "slight (1)" or "negative (0)". Subsequently, they were scored by multiplying the percentage of positive cells by intensity. This method is called "Quick Score".[41] the scale is ordinal.

4.7 RESEARCH TOOLS AND MATERIAL

4.7.1 **Research Tools**

1 Cage of mice (44 x 35 x 20 cm), the same for each group

2 Plate

3 Syringe

4.7.2 Research Material

a. Halothane with dose 0,011 mg/weight of mice

b. Balb/C mice (male)

c. Food and drink for BALB/C Mice

d. Pure formalin

e. HE staining

f. Cytochrome P450 2E1 antibody

4.8 DATA COLLECTION AND ANALYSIS

4.8.1 Data collection

The study was conducted in Animal Laboratory at Gajah Mada University. After terminated the mice, the liver tissue of the mice were taken to do histopathology and immunohistochemistry examinations. The slides for those examinations were made at The Pathology Laboratory of RS. Dr. Sardjito Yogyakarta and read at The Pathology Laboratory of RSUP Dr. Kariyadi Semarang. Sample in this research were BALB/c Mice (male), in healthy condition and 6–10 weeks old, no infectious disease and the weight was between 29 – 33 grams. The number of the

mice were 30 mice, divided into five groups, each group consist of 6 mice. The first group was Control Group, which the mice were not exposed to halothane. Group 1 is the group whose mice were exposed to 0,011 mg/weight halothane 3 hours daily for two (2) weeks. Group 2 had extended time of halothane exposure until four (4) weeks and Group 3 had for six (6) weeks of exposure. Group 4 was equal to the Group 3 but the mice were terminated after 2 weeks of free halothane.

Adaptation time was done in one day to prepare the mice, that is weighing, examining the health status, and help the mice to addapt with new environment. The dose for halothane was 1.2 ml per cage, given 3 hours daily for 2 weeks, 4 week, 6 weeks and 6 weeks follow by 2 week halothane free. The halothane was put on a plate for each cage then placed inside. The halothane used was liquid halothane manufactured by Nicholas Piramal India Limited. The halothane is an inhalation anesthetic that can evaporate easily at room temperature. Therefore, we just put it on a plate and keep inside of the cages and let the mice inhale it for 3 hours each day.

After halothane exposure for 2 weeks, the mice from the Group 1 were terminated. Initially, the mice were given halothane intravenously, then were operated to take its liver tissue. The liver were placed onto pure formalin for 24 hours, then processed by making parafin block. This procedure is standart procedure to take the liver organ.

The tissue on this parafin block was sliced using microtom. HE staining was performed as regular staining to explore the tissue changes and immunohistochemistry staining was undertaken to explore the cytochrome P450 in the liver by using cytochrome P450 2E1 antibody.

4.8.2 Data Analysis

Data collected from observation of liver cells changes and cytochrom P450 changes were descriptively analysed by counting the standard deviation and median. Numbers of liver cells changes and cytochrom P450 changes of each group were showed in box-plot graph.

Shapiro-Wilk test was performed to test the data normality. Subsequently, the hypothesis test was conducted to find whether there is any difference of liver cells and cytochrome P450 changes between the control group and the experimental group with different time of halothane exposure by Kruskall-Wallis test. If the difference was significant post hoc analysis was carried out by Mann-Whitney test. All the analysis were done using SPSS version 12.0 for windows.

4.9 Flow Chart of The Study

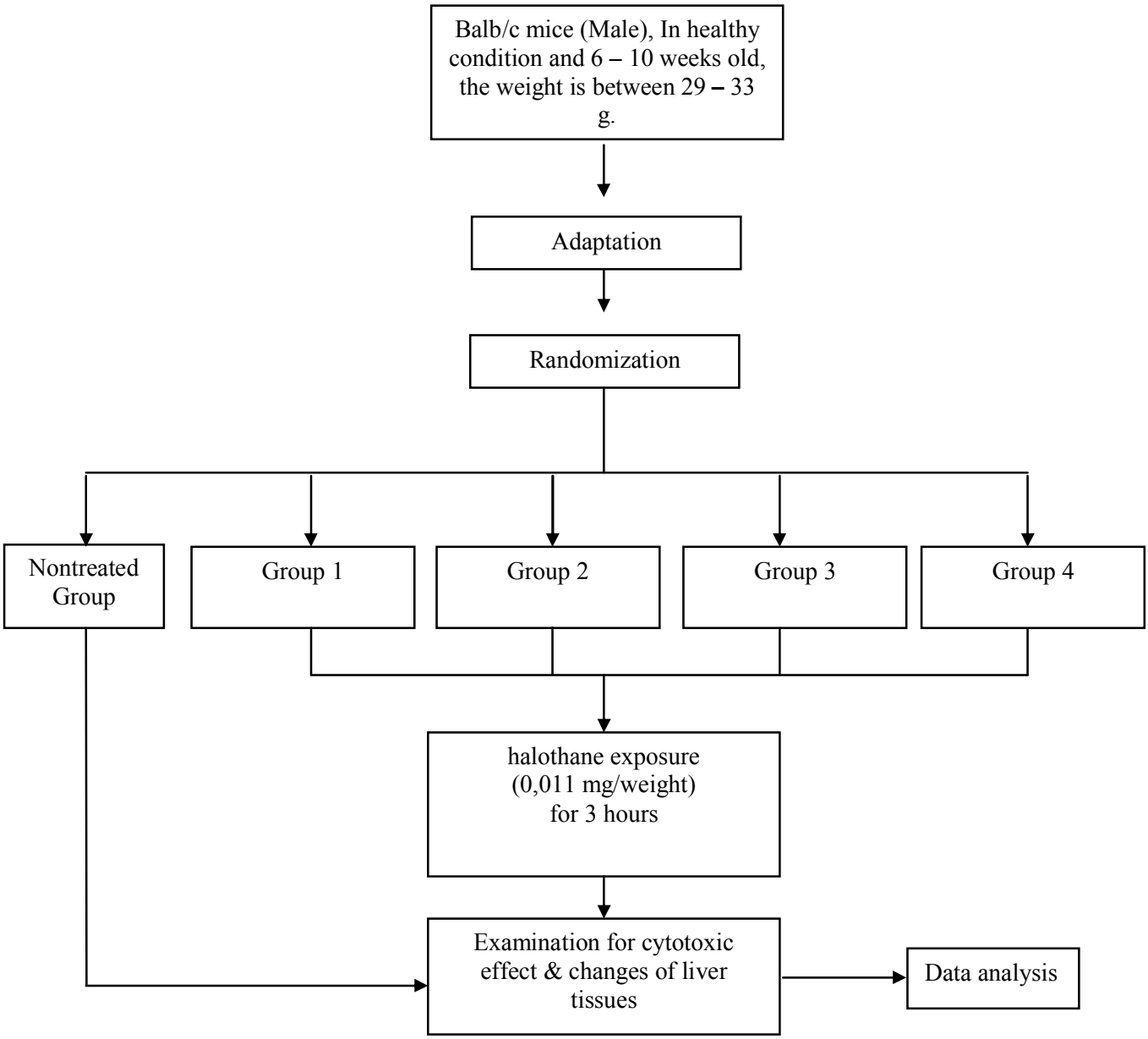

Fig 5. Flow chart of the study