

## CHAPTER IV

### RESEARCH METHODS

#### IV.1. Scope of the study

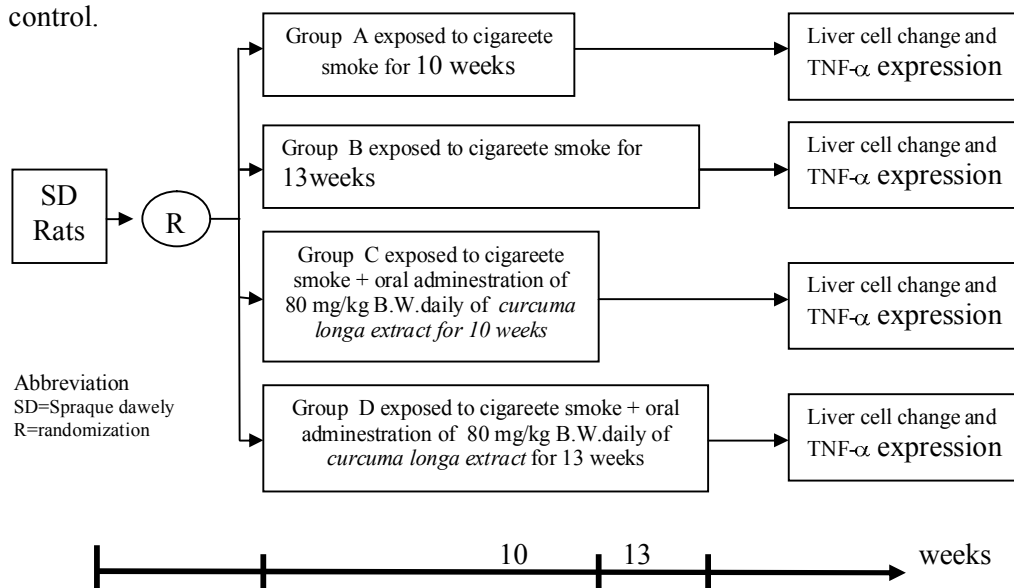
Scope of study was pathology anatomy ,theraby , histopathology and specially in the field of pathobiology.

#### IV.2. Place and time of the study

The study was conducted in Animal Laboratory at Gajah Mada University and the tissue examination was done at Pathology Laboratory of RSUP Dr. Kariyadi Semarang between Agust-October 2011.

#### IV.2 Research Design

Study design is experimental study,namely *post test only control group design*. The sample were randomly divided into 2 intervention group and 2 group for control.



### **IV.3. Subjects of the study**

Subjects of the study were Sprague Dawley albino rats inbred strain. The rats were obtained from Animal Laboratory at Gajah Mada University. Criteria of samples as follow:

#### **IV.3.1. Inclusion Criteria**

- Male
- Age 4 weeks, body weight was between 40-65g.
- Healthy characterized by active moving and no sign anatomic anomaly by inspection

#### **IV.3.2. Exclusion Criteria**

- Rats had diarrhea during the study, the activity looks abnormal , sneezing and semi-dormant
- Rats were death during experiment

#### **IV.3.3. Sampling methods**

Rats were allocated into 4 experiment group by simple random sampling using computer generated random number.

### **IV.4. Sample Size**

Sample size of the study was determined according to WHO requiriement for traditional medicine study. According to WHO guideline minimal 5 rats is necessary

in each group. In this study according to *Fredreic test* the sample in each group were 6 SD rats. The total animals were 24 rats.

$$\mathbf{Fredreic\ test} = \boxed{\mathbf{(T-1) (N-1) > 15}}$$

Abbreviation:  
**T**= number of the group  
**N**= number of the rats

$$\begin{aligned} &= \mathbf{(4-1) (N-1) >15} \\ &= \mathbf{(3) (N-1) >15} \\ &= \mathbf{(3N) - (3) >15} \\ &= \mathbf{(3N) < 15+3} \\ &= \mathbf{(N)_{<} 6} \end{aligned}$$

By using sampling techniques (simple random sampling) sampled as many as 6 tails

#### **IV.5. Study variables**

##### **IV.5.1. Independent variable**

Cigarette smoke and curcuma administration status:

- Cigarette smoke exposure for 10 weeks
- Cigarette smoke exposure for 13 weeks
- Cigarette smoke exposure followed by oral administration of *Curcuma L extract* 80 mg/kg B.W. daily for 10 weeks

- Cigarette smoke exposure followed by oral administration of *Curcuma L* extract 80 mg/kg B.W. daily for 13 weeks

#### IV.5.2. Dependent variables

- a. Liver cells change ( oedema cell ,karyiohexis ,karyiolysis )
- b. Liver tissue TNF- $\alpha$  expression score

**Table (2) Dependent variables definition**

No.	Variables	Scale
1.	<p><b>Cigarette smoke and curcuma administration status</b></p> <p>Cigarette smoke exposure is cigarette smoke exposure to animal that were put in closed chamber. The cigarette was commercially available filtered cigarette that obtained from local vendor. The dose was 5 cigarette /day were burned with 50 minute as Brak time (to prevent hypoxia of the Rats) between each cigarette for 10 weeks (group A) and 13 weeks (group C).</p> <p>Curcuma L extract is curcuma longa ethanol extract in the dose of 80 mg/kg (in 1 ml of 5% DMSO) daily administered using orally immediately after exposure to cigarette smoke for 10 weeks (group C) and 13 weeks (group D). Curcuma L extracts were purchased from Biotechnology laboratory of Gajah Mada University</p>	
2.	<p><b>Liver cells change</b></p> <p>Liver tissue change was determined by histopathology examination. Liver nucleus cell change were determined by percentage % as follow:( Oedematous cell , piknotic ,karyolysis of nucleous)Magnification was 400 X, examinations were performed in 10 fields, twice replication in consecutive days by 2 independent observers. Sites of examination were chosed randomly. The total score for each change in the one rat was counted and divided into 10.</p>	Nomerial
3.	<p>Liver tissue TNF-<math>\alpha</math> expression was be measured by immunohistochemical method. TNF-<math>\alpha</math> expression was quantified in accordance to Allred score. Allred score was established using a 0–8 scale based upon the sum of a proportion score (percent of stained cells) and</p>	Ordinal

No.	Variables	Scale																																
	intensity score (weak, intermediate, and strong). The possible values of Allred score are: 0 – Allred 0*; 1 – Allred 2, 3, 4; 2 – Allred 5, 6; 3 – Allred 7, 8 (*Allred score 1 is not possible). each slide rated 10 field of view with magnification 400X.																																	
	<table border="1"> <thead> <tr> <th colspan="2">Proportion Score (PS)</th> <th colspan="2">Intensity Score (IS)</th> </tr> <tr> <th>Value</th> <th>Significance</th> <th>Value</th> <th>Significance</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>none</td> <td>0</td> <td>none</td> </tr> <tr> <td>1</td> <td>&lt;1%</td> <td>1</td> <td>weak</td> </tr> <tr> <td>2</td> <td>1- 10%</td> <td>2</td> <td>intermediate</td> </tr> <tr> <td>3</td> <td>10- 33%</td> <td>3</td> <td>strong</td> </tr> <tr> <td>4</td> <td>33- 66%</td> <td></td> <td></td> </tr> <tr> <td>5</td> <td>&gt; 66%</td> <td></td> <td></td> </tr> </tbody> </table>	Proportion Score (PS)		Intensity Score (IS)		Value	Significance	Value	Significance	0	none	0	none	1	<1%	1	weak	2	1- 10%	2	intermediate	3	10- 33%	3	strong	4	33- 66%			5	> 66%			
Proportion Score (PS)		Intensity Score (IS)																																
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0	none	0	none																															
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2	1- 10%	2	intermediate																															
3	10- 33%	3	strong																															
4	33- 66%																																	
5	> 66%																																	
	Allred score: PS + IS																																	

#### IV.7. Material and method

##### IV.7.1 Research Tools

- A. Commercially available light cigarette (Marlboro®) obtained from local vendor
- B. *Curcuma longa* extract in dose 80 mg/kg B.W.daily
- C. Sprague Dawley albino rats
- D. Closed chamber designed for cigarette smoke exposure
- E. Water
- F. General pellet food for rats
- G. TNF- $\alpha$  immunohistochemical staining kit (RND system, USA)
- H. Hematoxyllin (HE) eosin staining kit
- I. Formaldehyde solution for tissue fixation

#### **IV.7.2 RESEARCH Material**

- A.** Glass slides.
- B.** Light Microscope for examination of tissues.
- C.** Hot plate or Water bath .
- D.** Wash bottle.
- E.** Racks for slides.
- F.** Marker of marking of slides .
- G.** Microtome .
- H.** Hot plate or water bath .

#### **IV.7.3. Animal care**

All animal was be care on Animal Research Care Facility of Medicine Gajah Mada University. Adaptation time was done in one week to prepare the rats ,that was weighing ,examining the health status ,and help the rats to adapt with new environment . They were housed in plastic cages with filter tops under controlled conditions , and raised by balancing feeding at room temperature and constant wetness degree according to the alteration of day and night . The rats were divided into four groups of six rats each. All Groups received a normal diet of standard pellets.

### **IV.7.3 Data collection method**

All animal were injected by ketamine injection as ( anaesthesia ) through tail vein,Rats liver tissue were taken for histopathology and immunohistochemistry examination. The slide for those examination was made at Pathology Laboratory of RSUP Dr. Kariyadi Semarang. Rats in group (A) was exposed to the cigarettes Smoke in dose 5 cigarettes with 50 minute intervals as preak time (to prevent hypoxia of the Rats in the cage ) between each cigarettes smoke exposure for 10 weeks. Group (B) was exposed to the cigarette smoke in dose 5 cigarettes with 50 minute intervals as preak time between each cigarettes smoke exposure for 13 weeks. Group (C) was exposed to the cigarette smoke in dose 5 cigarettes with 50 minute intervals as preak time between each cigarettes smoke exposure and followed by oral administration of Curcuma L in dose 80 mg/kg B.W.daily for the 10 weeks. Group (D) was exposed to the cigarette smoke in dose 5 cigarettes with 50 minute intervals as preak time between each cigarettes smoke exposure and followed by oral administration of Curcuma L in dose 80 mg/day B.W. daily for the 13 weeks.

## **IV.8. RESEARCH PROCEDURE**

### **IV.8.1. Preparation of Curcuma L extract**

The coarse powder of Curcuma longa (50 gram) was extracted with ethanol (300ml) using a soxhlet apparatus for 48 h at 60°C. Obtained ethanolic extract was

dried under reduced pressure at a room temperature not exceeding 40° C. were Rats received 80mg/kg B.W. daily of curcuma longa rhizoma extract orally by feeding ,

#### IV.8.2 Preparation of Marlboro ciggarete smoking

A Marlboro cigaretttes were brought from a local shop were Nicotine with level is 1.5 mg on the baket , each group has been giving a total number of 5 cigaretttes with Dividing time up to 50 minutes between each cigarettte as preak time to prevent hypoxia of the Rats

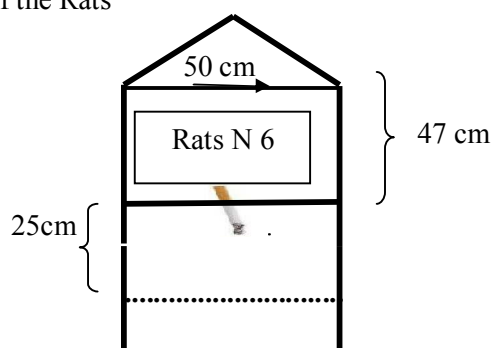


Fig 10. Design of the cage size of passive smoking

#### IV.8.3. Histology Specimen Preparation and staining (Liver cells change)

After the total duration of the experiment 10 weeks for (A&C),and 13 weeks for (B&D) at the end of it, the animals subjected to whole-body perfusion using normal saline and buffered formalin under light ether anesthesia. way to termination pay attention to the principles stated in Helsinki Declaration of 1975 and the National Guidelines for Health Research Ethics(PNEPK ),The liver removed



and stored immediately in buffered formalin for histopathology examination. The tissue fixed for at least 48 hours in buffered formalin. The specimens were dehydrated in ascending grades solutions of alcohol and cleared in xylene and then impregnating and embedding in paraffin wax for 2 hours . Sections cut at 5µm(Three pieces ) with the rotary microtome and then stained with hematoxylin and eosin for examination by light microscopy. <sup>11</sup>

Were the liver cells change is Liver Odeama Cell & Karyopiknotic or/and karyorhexis and Karyolysis

➤ **Odeamatus cell**

hepatocytes exhibited cloudy swelling with pale cytoplasm and poorly delineated and displaced nuclei . Cellular swelling might be accompanied by leakage of lysosomal hydrolytic enzymes that lead to cytoplasmic degeneration and macromolecular crowding

➤ **Karyopyknosis**

pyknotic nuclei is clumping and condensation of the chromatin materials in the periphery of the nuclei together with irregularity nuclear membranes Karyopyknosis ia an irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis

➤ **Karyolysis**

this alteration appeared as the complete dissolution of the chromatin matter of a dying cell

#### **IV.7.3. Liver tissue TNF- $\alpha$ expression immunohistochemistry procedure**

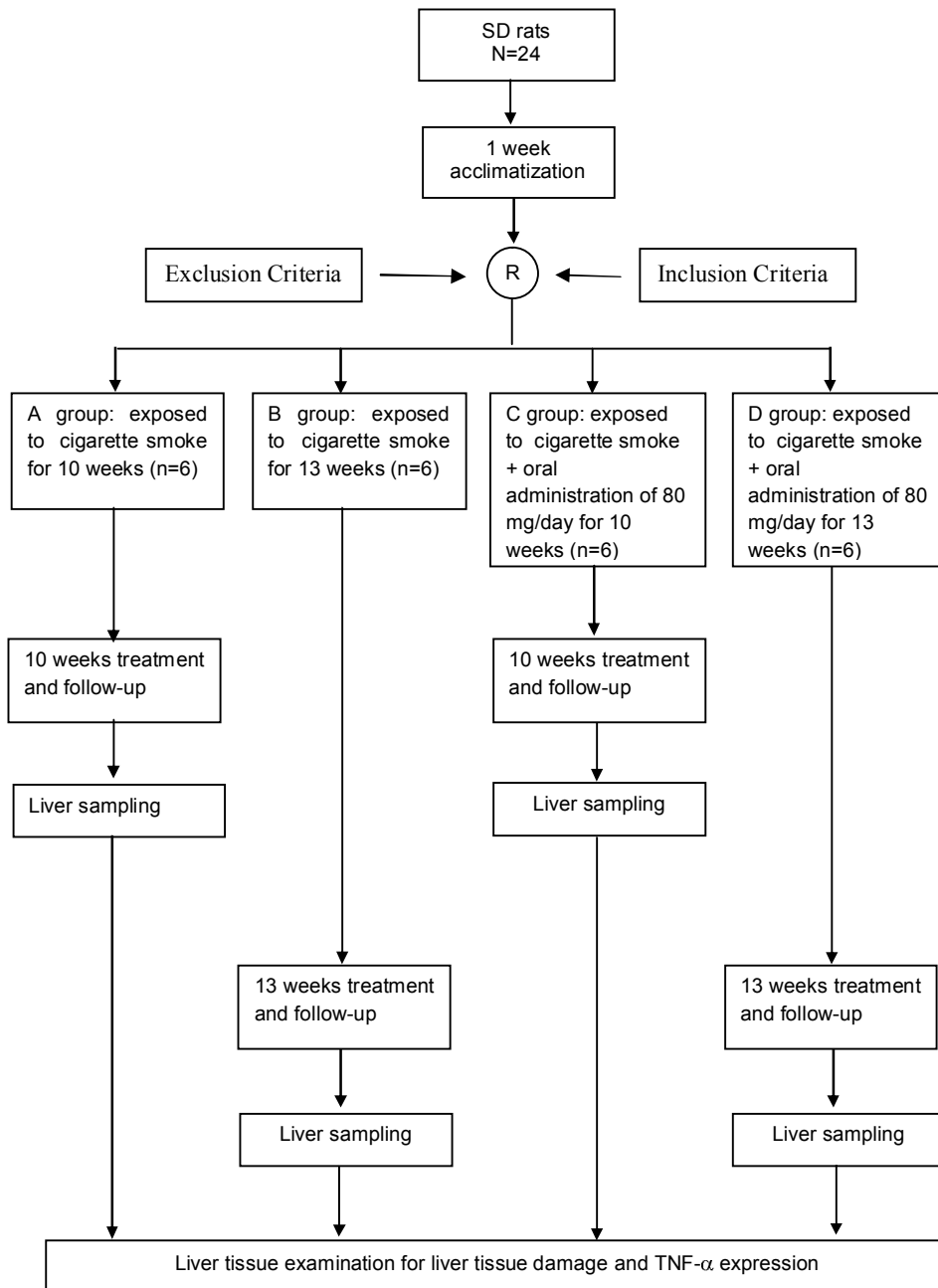
- Animals were sacrifice by overdose of ketamine injection.
- Liver was remove by clean surgery procedure.
- Liver tissue were fixed in formalin and embed in paraffin blocks according to standard procedures, were each slide got three pieces of the tissue section
- Object glass slides were cleaned with 95% ethanol and treated with subbing solution and air dry, or by using pre-treated slides.
- Tissue sections were cut 4–6 micron thick and applied to slides. Tissue were deparaffinize in xylenes using three changes for 5 minutes each. Hydrate sections gradually through graded alcohols: wash in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each. Wash in deionized H<sub>2</sub>O for 1 minute with stirring. Aspirate excess liquid from slides.
- Antigen unmasking was performed at this point. Certain antigenic determinants are masked by formalin fixation and paraffin embedding and may be exposed by Pepsin: Incubate sections for 10–20 minutes in 0.1% pepsin in 0.01 N HCl at room temperature. Slides were washed several times in deionized H<sub>2</sub>O. Aspirate excess liquid from slides.
- For immunoperoxidase staining of tissue sections, will use, ABC Staining Systems, The ABC Staining Systems utilize preformed avidin-biotinylated horseradish peroxidase complex as a detection reagent.<sup>52</sup>

- TNF- $\alpha$  immunohistochemical expression quantified in accordance to Allred score by two independent pathologists and compared across histological categories using Kappa test.

Were TNF- $\alpha$  is;

Tumor necrosis factor (TNF, cachexin or cachectin formerly known as tumor necrosis factor-alpha or TNF- $\alpha$ ) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by other cell types as well

#### IV.8. Flow of the study



**Fig. 11.** Flow chart of the study

#### **IV.9. Data analysis**

Before analysis data was checked for correct and completeness. Data were coded, tabulated and entry in to computer. At first, the data collected from examination of hepatic tissue changes in 5 fields for each slide were evaluated statistically by calculating median, then Kappa Statistic(K) was used to assess inter-pathologists agreement for the reliability and validity to each diagnostic scores. Number of liver hepatocyte changes of each group showed in box-plot graph

Data analysis was include descriptive analysis and test of hypothesis. Data of liver cell change and TNF- $\alpha$  expression score were expressed as mean and standard deviation (SD). Data normality distribution was checked by Saphiro-Wilk test. Saphiro-Wilk test showed liver cell change were normality distribute and TNF- $\alpha$  expression score were abnormally distributed. The difference of liver cells change score were analyzed by using independent t-test. P value < 0.05 is considered significant. and TNF- $\alpha$  expression score between A group and C group, B group and Dgroup were analyzed by using Mann-Whitney Test. P value < 0.05 is considered significant. Statistical analysis were performed by computer program.

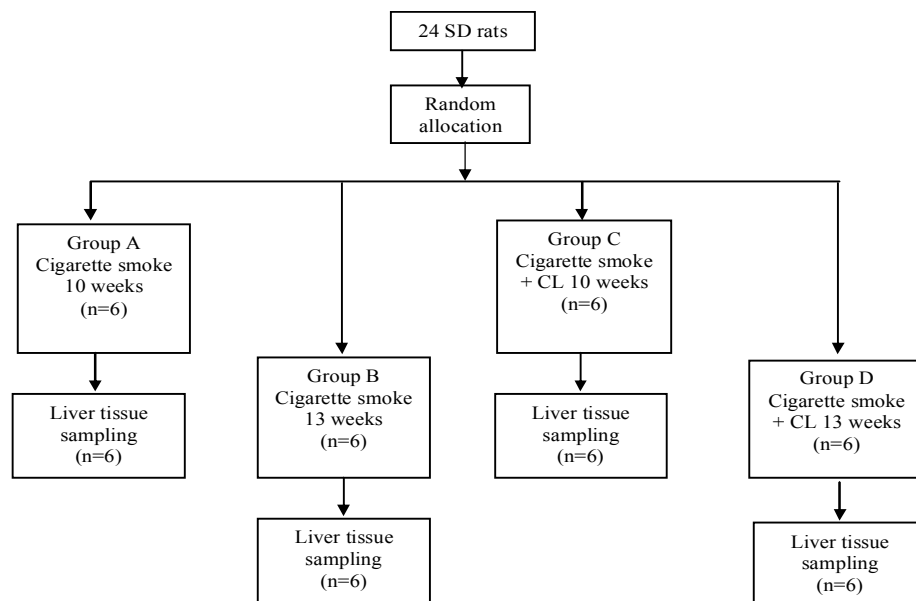
#### **IV. 10. Ethics**

Protocol of the study has been submitted to Ethical Committee of Medical and Health Research Medical Faculty Diponegoro University. Ethical clearance was obtained. All animal has been care according to Helsinki's Declaration. All invasive treatment have been conducted under anesthesia.

## CHAPTER V

### RESULTS

This study used 24 SD rats as study objects. Rats were randomly allocated by simple random sampling into 4 groups: Group A, rats were exposed to smoke of 5 cigarettes for 10 weeks (n=6); group B, rats were exposed to smoke of 5 cigarettes for 13 weeks (n=6); group C, rats were exposed to smoke 5 cigarettes and followed by oral administration of 80 mg/kg B.W.daily for 10 weeks; group D, rats were exposed to smoke 5 cigarettes and followed by oral administration of 80 mg/kg B.W. daily for 13 weeks. During study period no rats were sick or death. At final experiment all rats were used for data analysis. The number of rats during study period as showed on figure 12.

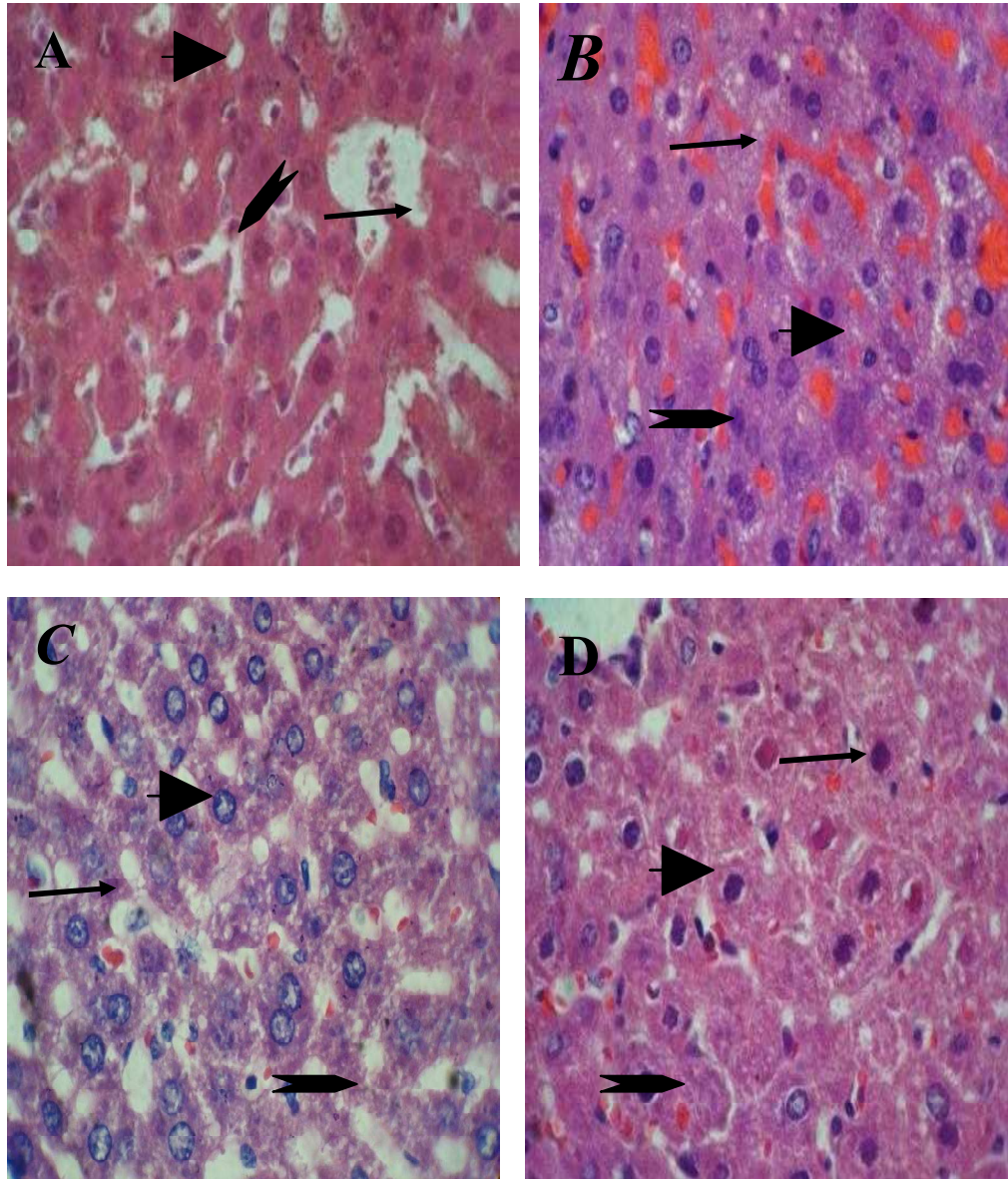


**Figure 12.** Animal number on study groups from the beginning until the end of study

#### 4.1. LIVER CELLS CHANGES

Histopathology feature of liver tissue on study groups at the end of experiment were shown on figures below. Tissue damage were observed from H&E staining examination by Olympus PX51 light microscope with 400x magnification in 10 fields from randomized choosing. The examiner counted the number of liver cells changes (i.e, odeama cell, karyopiknotic, and karyolysis). The results were expressed in percentage (%) of abnormal cells per all cells counted on those fields. From total 24 rats, 24 livers of the rats were made into tissue slide

Were the Rats distributed as group as ,Group A as control group was exposed to passive cigarette smoke for 10 weeks after that was terminated and liver of it was taken, Group B as control group was exposed to passive cigarette smoke for 13 weeks after that was terminated and liver of it was taken, Group C as treated group was exposed to passive cigarette smoke for 10 weeks followed by *curcuma longa rhizoma extract* after that was terminated and liver of it was taken, Group D as treated group was exposed to passive cigarette smoke for 13 weeks followed by *curcuma longa rhizoma extract* after that was terminated and liver of it was taken,



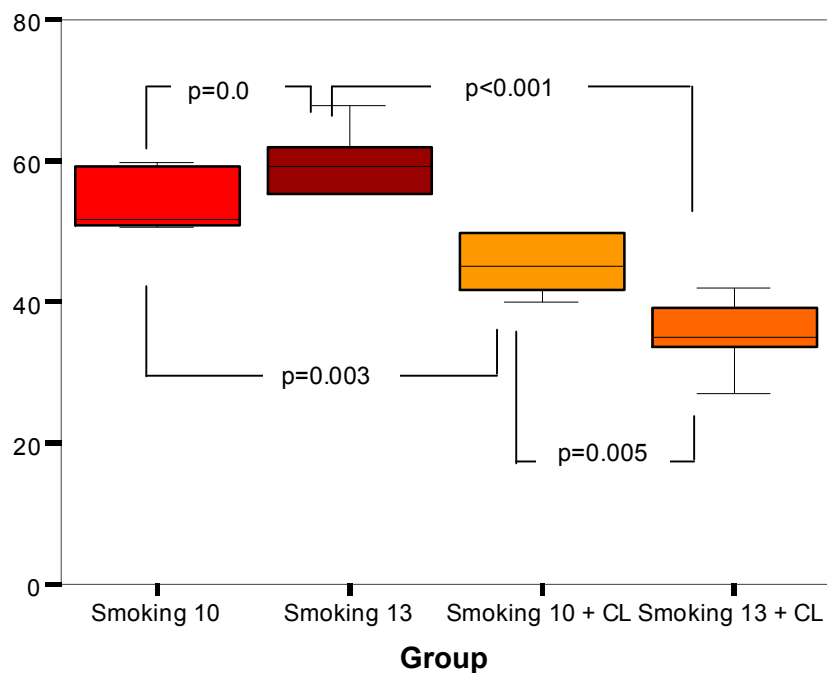
**Figure 13.** HE staining examination of group (A,B,C,D) (400X magnification) Histopathology feature of liver cells changes of SD rats after SD rats after 10 weeks (A)13 weeks (B )cigarette smoke exposure only,10 weeks cigarette smoke exposure + Curcuma L (C). 13 weeks cigarette smoke exposure + Curcuma L (D). ( Head Arrow) point cell with odeama ,(tailed arrow) karyolysis and karyopiknotic (long thin arrow) ,



Figure 13 showed large number of liver cells with liver cells change such as oedema, karyolysis and karyopicnotic on SD rats that exposed to cigarettes smoke for 13 weeks, in other hand, on SD rats that exposed to cigarette smoke with *Curcuma L* administration for 10 weeks , the number of cells with liver cells changes were smaller , Liver cell change score on study groups were showed on tabel 3.

**Table 3.** Liver cells change on study groups (n=24)

Group	Liver cells change ( Oedema Cell &Karyolysis and Karyopiknotic %)	P /Value
	Mean $\pm$ SD; Median (Min - Max )	
Smoking 10 Group (A)	50.9 $\pm$ 6.40; 48.8 (45.3 - 57.8)	A&B=0.01
Smoking 13 Group(B)	73.9 $\pm$ 10.07; 75.0 (56.5 - 85.6)	A&C=0.003
Smoking 10 + Curcuma L. (C)	43.3 $\pm$ 1.92; 42.9 (41.4- 45.7)	B&D=0.001
Smoking 13 + Curcuma L. (D)	53.7 $\pm$ 5.57; 52.7 (51.6 - 62.0)	D&C=0.005



**Figure 14** Box plot diagram of liver cells change score on study groups. P value were obtain from Boferroni post hoc test.

Table 3 showed the mean of liver cells change score on SD rats on study groups. The highest score was in group (B) 13 weeks cigarettes smoke exposure group, followed by group (D) 13 weeks cigarettes smoke exposure group + *Curcuma*, group (A) with 10 weeks smoke exposure and the lowest was group (c) 10 weeks cigarette smoke exposure + *Curcuma L*. The difference of Liver cells change score between groups were statistically significant ( $p=0.01$ ). Figure 14, show the comparison of liver cells change score of each study group. Multiple comparison by Bonferroni post hoc test showed liver cells change score of 10 weeks exposure cigarette smoke + *Curcuma L* was significantly lower than 10 weeks exposure of cigarette smoke only ( $p=0.003$ ). Furthermore, liver cells change score of 13 weeks exposure cigarette smoke + *Curcuma L* was significantly lower than 13 weeks exposure of cigarette smoke only ( $p<0.003$ ). On Figure also was showed liver cells change score of 13 weeks cigarette smoke exposed group was higher than 10 weeks exposure, however the difference was statistically significant ( $p=0.01$ ). In contrary, the liver cells change score of 13 weeks cigarettes smoke exposure + *Curcuma L* was significantly lower than 10 weeks cigarette smoke exposure + *Curcuma L* ( $p=0.005$ ). These results may also can be interpreted as longer administration of *Curcuma L* may give bigger liver cells change score reduction.

Base on those findings the hypothesis that *curcuma L* administration of *Curcuma L* have potency to reduce liver cells nuclear change due to cigarette smoke exposure for 10 weeks and 13 weeks has been proven.

## V.2. Liver cells TNF- $\alpha$ expression

Expression of TNF- $\alpha$  observed by immunohistochemistry examination using TNF $\alpha$  antibody ,examination of slide done by Olymps PX51 light microscope using 10X ocular lens and 40X objective lens, examination and reading of the slides were done by counting the percentage of TNF- $\alpha$  cells stained brown color in the cytoplasm as proportion score adding to it the intensity of the staining rated as none, mild, intermediate and strong, the result of the these two score is a number which is called Allred score into: : 0 – Allred (0\*); 1 – Allred (2, 3, 4); 2 – Allred (5, 6); 3 – Allred ( 7, 8). Liver tissue TNF- $\alpha$  expression on study groups were showed on figure 15 , showed large number of liver cells that expressed TNF- $\alpha$  on 10 and 13 weeks cigarette smoke exposure. In another hand, the number of liver cells that expressed of TNF- $\alpha$  on 10 weeks passive smoke exposure + *Curcuma L* and 13 weeks passive smoke exposure + *Curcuma L* look smaller than without *Curcuma L*. administration on Figure 15

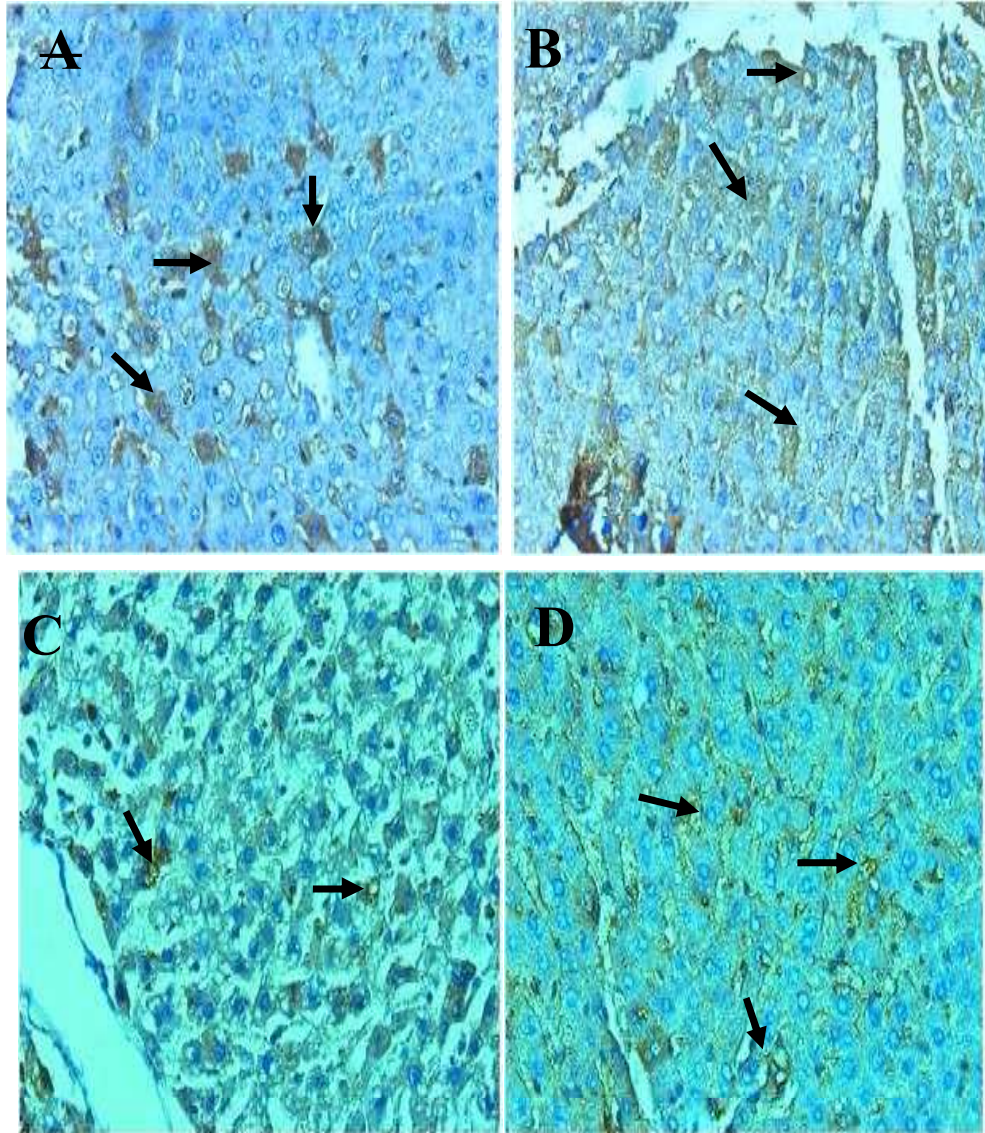
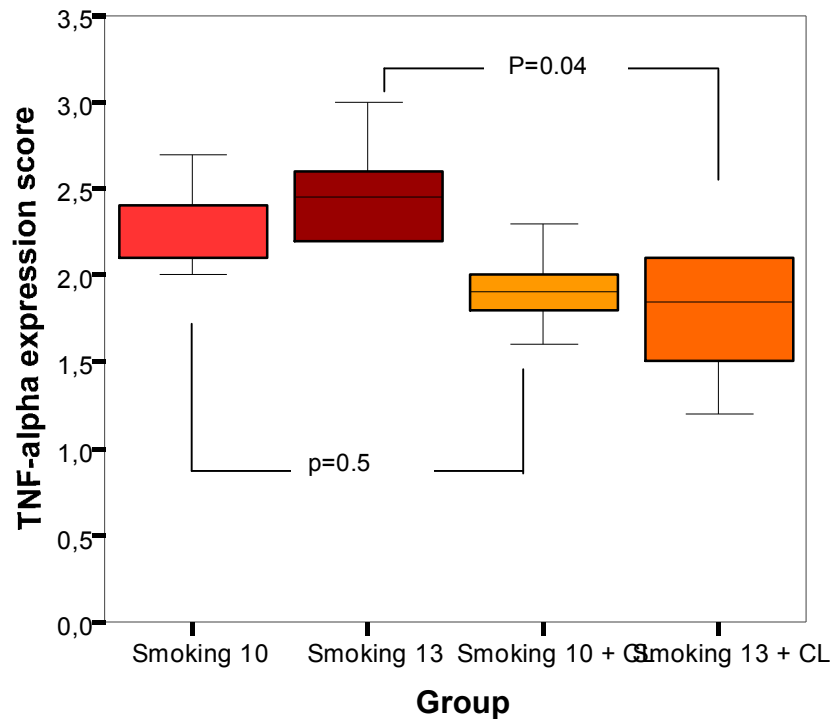


Figure 15. Morphology of TNF- $\alpha$  expressions of liver tissue of SD after 10 weeks for group A cigarette smoke exposure, 13 weeks for group B cigarette smoke exposure, 10 weeks for group C cigarette smoke exposure + Curcuma L, 13 weeks for group D cigarette smoke exposure + Curcuma L using 400X magnification, Arrow point to liver cells that expressed TNF- $\alpha$ ,

Table 4 showed the highest TNF- $\alpha$  expression of liver cells was found on 13 weeks cigarettes smoke exposure group, followed by 10 weeks cigarettes smoke exposure, 10 weeks cigarettes smoke exposure + *Curcuma L* and the lowest was 13 weeks cigarettes smoke exposure + *Curcuma L*.

**Table 4.** Liver cells TNF- $\alpha$  expression score in study groups (n=24)

Group	TNF- $\alpha$ expression score Mean $\pm$ SD; Median (Min - Max)	Sig P/Value
Smoking 10 Group (A)	2,0 $\pm$ 0,63; 2,00 (1 - 3)	A&C=0,5
Smoking 13 Group (B)	2,25 $\pm$ 0,75; 2,25 (1 - 3)	C&A=0,5
Smoking 10 + Curcuma L (C)	1,83 $\pm$ 0,40; 2,00 (1 - 2)	B&D=0.04
Smoking 13 + Curcuma L (D)	1,8 $\pm$ 0,75; 2,00 (1 - 3)	D&B=0.04



**Figure 16.** Box plot diagram of liver tissue TNF- $\alpha$  expression score on study groups. P value were obtain Mann-Whitney Test post hoc test.

Figure 16, showed liver tissue TNF- $\alpha$  expression of group (C) 10 weeks cigarettes smoke exposure + *Curcuma L* group was lower than group (A) 10 weeks cigarettes smoke exposure only group, however, the difference was not significant ( $p=0.5$ ). The liver tissue TNF- $\alpha$  expression of Group (D) 13 weeks cigarettes smoke exposure + *Curcuma L* was significantly lower than Group (B) 13 weeks cigarettes smoke exposure only ( $p=0.004$ ). Figure 22 also showed liver tissue TNF- $\alpha$  of 13 weeks cigarettes smoke exposure group was higher than 10 weeks exposure, however, the difference was more as median (2,25). This findings suggest with increasing period exposure of cigarettes smoke that cause increase of liver tissue TNF- $\alpha$ , longer exposure time was supra-maximal exposure time. Longer administration of *Curcuma L* may give bigger reduction of liver tissue TNF- $\alpha$  expression.

Base on those findings the hypothesis that administration of *Curcuma L* have potency to reduce up-regulation of liver tissue TNF- $\alpha$  expression due to cigarette smoke exposure for 13 weeks has been proven. On the other side the administration of the *curcuma L* didnt have potency to reduce up-regulation of liver tissue TNF- $\alpha$  expression due to cigarette smoke exposure for 10 weeks may give suggest that with increasing time using of *curcuma L* inhance the regenirative role aganist the expusore effect of passive cigarette smoke

Base on those findings the hypothesis in the tow variable given suggest that the regenireative role of the administration of *curcuma L* on the liver cell change is

more potency reduction than regenerative role on the TNF- $\alpha$  expression may be because the curcuma longa extract act as antioxidant much more than act as anti-inflammatory production and also may be because curcuma longa extract can be regenerative role much more against early stage of liver damage like liver cell change than than serious liver cell damage like TNF- $\alpha$  expression as inflammation proces inside liver cell at the same period of the curcuma longa administration

#### **V. Reliability of measurement:**

The current study calculated reliability coefficient of scores by measuring agreement between two observers, using Kappa coefficient test, which examines the agreement between two raters for a sureness whether there is a concordance in reading the data, the table below shows the result of Kappa test for Liver cells change and TNF $\alpha$  scores. significant at level ( $\alpha = 0.05$ ).s

According to table(Appendix 3) there is agreement between both of reading , because the values of chi square (16.7 & 0.22) were not significant in two variables (tliver cells change and TNF $\alpha$ ) the p values were more than (0.05), and that means the measurement was reliable.