CHAPTER III
RESEARCH METHODS

3.1. Scope of study

Scope of study is biomedical especially immunology.

3.2. Place and time of the research

The study had been done in Laboratorium Penelitian dan Pengujian Terpadu (LPPT) at Gadjah Mada University and the blood examination was done in the Pathology Department Gadjah Mada University Yogyakarta. Precisely this study was done for two months starting from January 2014 until March 2014.

3.3. Research Design

Study design is experimental study, by using randomized post-test only control group design. Treatment by giving extra virgin olive oil and outcome eNOS and ICAM-1
Notes:
R : random allocation in 5 groups
K1 : negative control
K2 : positive control
OK1 : outcome of negative control
OK2 : outcome of positive control
XT1 : treatment group I (high fat diet and EVOO dosage I: 1 ml/KgBW for 60 days)
XT2 : treatment group II (high fat diet and EVOO dosage II: 2 ml/KgBW for 60 days)
XT3 : treatment group III (high fat diet and EVOO dosage III: 3 ml/KgBW for 60 days)
OT1 : outcome from treatment group I
OT2 : outcome from treatment group II
OT3 : outcome from treatment group III

3.4. Population and Sample

3.4.1. Population

Population of this study were Rattus Novergicus Strain Wistar rats

3.4.1.1 Inclusion Criteria

a) Rattus novergicus strain Wistar rats, male, activity, health.

b) Weight (150-200 g).

b) Age 8 weeks.

3.4.1.2 Exclusion Criteria

a. Visible anatomic abnormality

b. Get diarrhea before intervention
3.4.1.3 Drop out

Rats death during experiment.

3.4.1.4 Randomization

The rats were randomly allocated into study group by simple random sampling using random number table.

3.4.1.5 Sample size

Sample size of this study was calculated by using Federer formula as follow:

\[
(n-1)(t-1) \geq 15
\]

Notes

\[
\begin{align*}
  n & \text{: total rats} \\
  t & \text{: treatment}
\end{align*}
\]

Calculation:

\[
(n-1)(t-1) \geq 15 \\
4n - 4 \geq 15 \\
4n \geq 19 \\
n \geq 5 \rightarrow \text{total rats: 25 rats}
\]

According to WHO 1993, minimal sample size for each group is 5 samples. One rat was added to all groups as spare if drop out is occured. In this study there were 5 groups, so total rats: 30 rats.

3.5. Variables of Study

3.5.1. Independent Variables

a. High fat diet.

b. Extra virgin olive oil (EVOO) in different doses.

3.5.2. Dependent Variables

a. Serum level of eNOS

b. Serum level of ICAM-1
3.6. Operational Definition

Table 2: Operational definition

<table>
<thead>
<tr>
<th>No</th>
<th>Variables</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Extra virgin olive oil (EVOO) in different doses</strong></td>
<td>Nominal, five group</td>
</tr>
<tr>
<td></td>
<td>Doses of EVOO are 1ml, 2ml, 3ml/KgBW for 60 days.</td>
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<tr>
<td></td>
<td>EVOO was administered orally 1 times daily in the morning</td>
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<tr>
<td>2</td>
<td><strong>ICAM-1</strong></td>
<td>Rasio</td>
</tr>
<tr>
<td></td>
<td>Serum level of ICAM-1 was measured using ELISA method.</td>
<td></td>
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<tr>
<td></td>
<td>Unit: ng/mL</td>
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<tr>
<td>3</td>
<td><strong>eNOS</strong></td>
<td>Rasio</td>
</tr>
<tr>
<td></td>
<td>Serum level of eNOS was measured using ELISA method.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unit: ng/mL</td>
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</tbody>
</table>

3.7. Data Analysis

Before doing analysis the data had been checked for the correction and completeness. Data were coded, tabulated and entered into computer.

Data analysis was included descriptive analysis and test of hypothesis. On descriptive analysis data in continuous scale such as eNOS level and ICAM-1 level were presented as mean and standard deviation (SD). Since sample size is less than 50, data distribution was checked by Saphiro-Wilk test.
The difference of eNOS and ICAM-1 serum level between control (-), control (+) and treatment I, II, III group was analyzed by ANOVA test followed by Bonferroni Post hoc test if data are normally distributed. P value < 0.05 was considered significant.

Statistical analyses were performed by SPSS program version16.

3.8. Research Instrument and Substance

3.8.1. Instruments

a. Instruments used for making diet were: scale, analytical scale, cup, and glass.

b. Instruments used for caring rats were: plastic container 45 cm x 35,5 cm x 14,5 cm, iron container 36,5 cm x 28 cm x 15,5 cm, bottle.

c. Scale was used to observe rats weight.

d. ELIZA microplate reader (Shimadzu, Japan).

3.8.2. Material

a. Rats nowergicus strain Wistar rats, male

b. EVOO from commercial vendor

c. Normal diet: confeed PARS, flour, and water

d. High fat diet: confeed PARS, flour, cholesterol, cholic acid, and water

e. Rats serum collected through rat eye vein

f. Specific kit for eNOS measurement (Quantikine, USA)

g. Specific kit for ICAM-1 measurement (Quantikine, USA)
3.8.3. Experiment methods

a. Before doing treatments all rats were put in individual cage. The temperature was about 25-30° C and humidity was about 20-30% with day and night simulation light system

b. All rats were adapted for 7 days before experiment

c. Food and water were delivered ad libitum

d. Rats were randomly allocated into 5 groups:
   1. Group with normal diet
   2. Group with high fat diet
   3. Group with high fat diet + EVOO dosage I
   4. Group with high fat diet + EVOO dosage II
   5. Group with high fat diet + EVOO dosage III

e. Normal diet composition: confeed PARS 225 gram, flour 100 gram, and water 100 ml. Total diet 40 gram/rat

f. High fat diet composition: additional cholesterol 2%, cholic acid 0,2%, and pig oil 5%. This is the highest fat diet composition: confeed PARS 200 gram, flour 100 gram, cholesterol 8 gram, cholic acid 0,8 gram, pig oil 40 ml, water 51,2 ml. Total high fat diet 40 gram/ rat

g. Olive oil dosage: Extra virgin olive oil doses used in this study were 1mL, 2 mL, 3mL /kg /day for period of 60 days

h. The food of all rats were scaled to know the intake of rats/ day.

i. The rats were randomly allocated into 5 groups
j. During treatment, rats in negative control group were feed by normal diet, rats in positive control group were feed by high fat diet, rats in treatment group were given high fat diet and EVOO dosage I, II, III

k. At the end of experiment, blood was collected from vein tail, put in the heparinized vacutainer. Blood sample was centrifugated immediately to collect serum for eNOS and ICAM-1 measurement

l. Measurement variable eNOS and ICAM-1.

m. Measurement of eNOS and ICAM-1 were performed as described in the kit measurement manual (attached).
3.9. Research Flow

Rats were adapted for 7 days by giving normal diet

Weight scale

Meet inclusion Criteria

Exclusion Criteria

Study objects

Randomization

Rats were divided into 5 groups

Control –  Control +  HFD+EVOO I  HFD + EVOO II  HFD + EVOO III

Treatment for 60 days

Blood sample collection

Measurement of serum levels of eNOS and ICAM-1

Data analysis and report writing
3.10. Research Ethics

Protocol of the study was submitted to Ethical Committee of Medical and Health Research Medical Faculty Diponegoro University for ethical clearance. The study was started after ethical clearance was obtained.

All animals were cared according to Helsinki’s Declaration. All invasive treatments had been conducted under anesthesia. The animals were killed as soon as possible under anesthesia after evaluation finished.