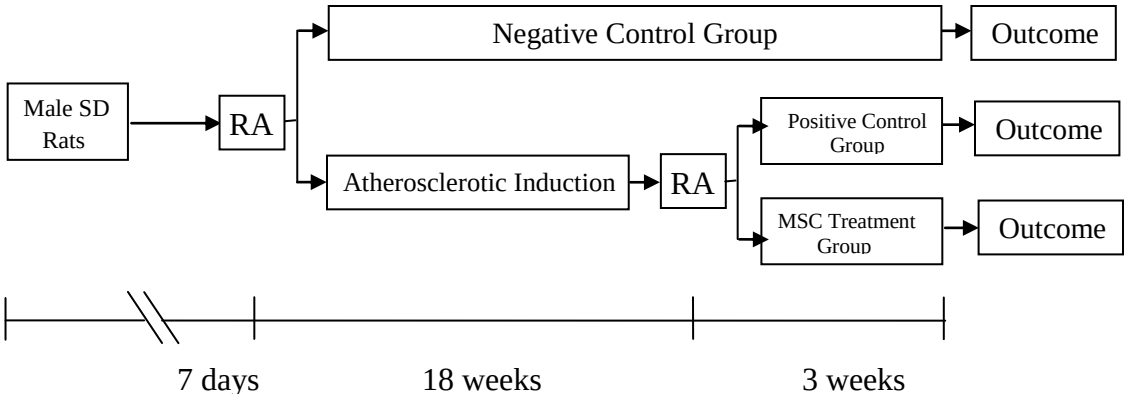
CHAPTER 4

RESEARCH METHOD

4.1. Research Design

This is an experimental study with *Randomized Post-test Only Control Group Design*. Samples were twice randomly allocated into 3 groups: negative control group, positive control group, and MSC treatment group. The scheme of research design is as follow:



Note:

Male SD Rats : Male Sprague Dawley Rats.

RA : Random allocation.

Atherosclerotic Induction : Single dose vitol-140® and eighteen weeks atherosclerotic diet

Negative control group : Standard diet all along the study.

Positive control group : Three weeks standard diet after atherosclerotic induction

MSC Treatment group : Single dose intra vena mesenchymal stem cell injection, followed by three weeks standard diet after atherosclerotic induction

4.2. Research Population and Sample

4.2.1. Population of Study

Populations of the study were male Sprague Dawley rats from Research Animal Developmant Unit inbreded in Private Ressearch Animal Development, Sleman, Yogyakarta.

Sample criteria as follow:

4.2.1.1. Inclusion Criteria

1. Male Sprague Dawley rats
2. Age ± 4 month
3. Body weight 200±50 gram
4. Healthy: move actively, no appearance of anatomic malformation, and no sign of infection.

4.2.1.2. Exclusion Criteria

1. Diarrhea that be signed by unshaped feces

4.2.2. Sample Size

Determination of the sample size was based on World Health Organization (WHO) guideline of animal sample requirement for medicine study, at least 5 animals on each group of study. To ensure sufficiency, this study used 6 male SD rats for negative control group, 4 male SD rats to make sure atherosclerosis in SD rats, 6 male SD rats for

positive control group, and 8 male SD rats for MSC treatment group. So, total samples were 24 male SD rats.

4.3. Randomization

Animals have been allocated into 3 groups by twice simple random sampling. Random number has been generated by computer. First randomization, each rat has been coded, and allocated into negative control group, and atherosclerotic induction group. After the rats developed atherosclerosis, atherosclerotic induction group was randomly allocated into positive control group, and MSC treatment group according to the obtained random number.

4.4. Research Variable

4.4.1. Independent Variable

Independent variable for the study was the administration of mesenchymal stem cell from umbilical cord matrix of white Sprague Dawley rats.

4.4.2. Dependent Variable

The Dependent variables for the study were:

1. Atherosclerotic plaque
2. TGF-β1 expression
3. IL-1α expression
4. IL-6 expression

4.4.3. Restrained Variable

Restrained variable for the study were:

1. Sex
2. Gene
3. Weight
4. Health condition
5. Woof
6. Cage

4.5. Operational Definition

4.5.1. Umbilical cord matrix mesenchymal stem cell

MSCs are progenitor that gives rise to multiple mesodermal derivatives such as bone, muscle and fat. MSC was isolated from umbilical cord of 19-20 days pregnant SD rats. It was cultured and subcultured until passage 4. MSC of passage 4 in dose 5 x 106 (in 1 ml NaCl) was injected to tail vein of atherosclerotic SD rats.

Scale: Nominal

4.5.2. Atherosclerotic plaque

Atherosclerotic plaque is vessel damage which signed by endothelial disfunction, foam cell formation, smooth muscle migration, fibrous cup formation and calsium plaque formation. Atherosclerotis plaque of abdominal aorta was measured by hematoxilin and eosin staining.

Scale: Ordinal

Atherosclerotic plaques were quantified in accordance to modified score.

Table. 4.1. Modified atherosclerotic score

Atherosclerotic Score	
Value	**Significance**
1	Foam cells
2	Progression
3	Advance

4.5.3. IL-1α expression

IL-1α is a protein of the interleukin-1 family that encoded by the IL-1α gene. IL-1α expression in vessel wall (endothel, smooth muscle and macrofage) of abdominal aorta was measured by immunohystochemistry.

Scale: Ordinal

4.5.4. IL-6 expression

IL-6 is a 26-kDa cytokine produced by vascular endothelial cells, mononuclear phagocytes, fibroblasts, activated T lymphocytes, and various neoplasms such as cardiac myxomas, bladder cancer, and cervical cancer. IL-6 expression in vessel wall (endothel, smooth muscle and macrofage) of abdominal aorta was measured by immunohystochemistry.

Scale: Ordinal

4.5.5. TGF-β1 expression

TGF-β1 is a polypeptide member of the transforming growth factor beta superfamily of cytokines encoded by TGF-β1 gene. TGF-β1 expression in vessel wall (endothel, smooth muscle and macrofage) of abdominal aorta was measured by immunohystochemistry.

Scale: Ordinal

TGF-β1, IL-1α and IL-6 were quantified in accordance to modified intensity score.

Table. 4.2. Modified intensity score

Intensity Score	
Value	**Significance**
0	None
1	Weak
2	Moderate
3	Strong

4.6. Material and Method

4.6.1. Animal Care and Atherosclerotic Induction

4.6.1.1. Animal Research Tools

1. Individual rat cages
2. Animal weight
3. Woof container

4. Water drink container
5. Disposable syringe

4.6.1.2. Animal Research Material

1. 24 male Sprague Dawley rats from Research Animal Developmant Unit inbreded in Private Ressearch Animal Development, Sleman, Yogyakarta, according to inclusion criteria.
2. Rodent standard diet (SD) AIN-93 M from Department of Pharmacology and Therapy, Faculty of Medicine, Gadjah Mada University. AIN-93 M composition is showed in Table 4.2.
3. Rodent atherogenic diet (AD) AIN-93 G from Department of Pharmacology and Therapy, Faculty of Medicine Gadjah Mada University. AIN-93 G composition is showed in Table 4.2.

   We add AIN-93 G with 10% cow oil in the first week of atheroclerotic induction, 20% cow oil in the second week of atheroclerotic induction, 30% cow oil in the the third week of atheroclerotic induction and 40% cow oil from the fourth week until the eighteenth week of atheroclerotic induction.

Table 4.3. Diet composition

Composition	AIN-93 M	AIN-93 G
Maizena	62 %	53.3 %
Casein	14 %	20 %
Sucrose	10 %	10 %
Happy salad	4 %	7 %
Jelly	5 %	5 %
Vitamin mix	1 %	1 %
Mineral mix	3.5 %	3.5 %
L-cystein	0.18 %	0.3 %
Cholin bitarate	0.25 %	0.25 %

4. Vitol-140® in dose 1.5 ml/100g body weight (120,000 IU vitamin A, 60,000 IU vitamin D3 and 30 mg vitamin E per 100 gram body weight).

4.6.1.3. Animal Care and Atherosclerotic Induction Method

1. Animal Care

   Animals-White Sprague-Dawley rats, male, weighing 200±50 gram, health, from Research Animal Development Unit inbreded in Private Ressearch Animal Development, Sleman, Yogyakarta, were maintained under controlled environment (28-320C), placed individually, enough ventilation, and provided standard diet (AIN-93 M) and water ad libitum. Cages were cleaned everyday to keep rats

healthy. All animals were acclimated a week before being used. Animal experiments were conducted strictly according to ethical guideline for using animal in scientific research and were conducted after the approval of the Medical Research Ethics Committee of Diponegoro University.

2. Diet for negative control group

   After aclimatization, white SD rats were fed with rodent standard diet AIN-93 M until termination.

3. Atherosclerotic Induction

   After aclimatization, white Sprague–Dawley rats (male, 200±50g body weight) were fed with atherogenic diet AIN-93 G plus 10% cow oil for the first week, AIN-93 G plus 20% cow oil for the second week, AIN-93 G plus 30% cow oil for the third week and AIN-93 G plus 40% cow oil for the forth to the eighteen week.

   The first day of the second week of atherosclerotic induction, single dose of vitol-140® 1.5 ml/100g body weight (120,000 IU vitamin A, 60,000 IU vitamin D3 and 30 mg vitamin E per 100 g body weight) was injected intraperitoneally.

4. Diet for positive control group

   After induction of atherosclerotic vessel wall, this group was fed with rodent standard diet AIN-93 M for 3 weeks.

5. Diet for MSC treatment group

   After induction of atherosclerosis, this group was injected with a single dose of MSC intravena and fed with rodent standard diet AIN-93 M for 3 weeks.

4.6.2. Mesenchymal Stem Cell Isolation

4.6.2.1. MSC Preparation Tools

1. Pincet
2. Scissor
3. Blade and scalpel
4. Petri dish
5. Micro pipets and tips
6. Tissue culture dish
7. Incubator CO2
8. Regulator and tank CO2

4.6.2.2. MSC Preparation Material

1. NaCl 0.9%
2. Phosphate-buffered saline (PBS)

3. MSC complete growth medium

   Composition each 100 ml of MSC complete medium:

   - DMEM low glucose 88,5 ml
   - Fetal bovine serum 10 ml
   - Pensrep 1 ml
   - Fungizone 0.5 ml

4.6.2.3. MSC Isolation Procedure

1. The umbilical cord was collected and stored in a sterile specimen cup containing 0.9% normal saline at 40C until processing.
2. The cord was handled in an aseptic fashion and processed in a Type II Biological Safety Cabinet.
3. The surface of the cord was rinsed with sterile phosphate buffered saline to remove as much blood as possible.
4. The length of the cord was estimated. The cord was manipulated in a sterile 10 cm petri dish.
5. The cord was cut as small as possible using a sterile blade.
6. 6-8 spots pulpy umbilical cord were placed to the 35 mm tissue culture dish.
7. Gently pipet 0.75 ml complete growth medium into each 35 mm tissue culture dish.

8. Incubated dish at 370C, 5% CO2 for 24 h.
9. After 24 h, explants were observed under inverted microscop, after the cells growth were found beside the explants, 2 ml complete medium were added.
10. After 48 h, cells and explants in the tissue culture dish were fed with fresh medium.
11. The cells were fed by the removal/replacement half of the medium every 2–3 days until the cells reach approximately 80% confluence.

4.1.1. Mesenchymal Stem Cell Growth and Subculture

4.1.1.1. Passaging The Cells

1. The cells were passaged when they are 80–90% confluent.
2. The medium was aspirated off and the cells are rinsed with sterile phosphate buffered saline (Ca2+ free).
3. A minimum amount of warmed, CO2-equilibrated trypsin–EDTA (0.05%) was added to the dish to cover the culture surface.
4. The dish was allowed to sit at room temperature for 1–2 min.
5. Then the detachment of the cells was observed under a microscope and detachment was facilitated by repeatedly tapping the dish gently on a hard surface.

6. The cells were not allowed to be in contact with trypsin–EDTA for more than 5 min.
7. The trypsinization reaction was neutralized by adding 2–3 times volumes of medium.
8. The solution containing the cells was transferred to a 15 ml sterile centrifuge tube and centrifuged at 2000 rpm for 10 min at room temperature.
9. The supernatant was discarded and the cells were resuspended gently in fresh medium.
10. The cells were counted and transferred to a new plate or flask at a concentration of 10,000 cells per cm2 in fresh medium.
11. The dish was incubated at 370C, saturating humidity and 5% CO2.
12. The dish was checked for confluence every day and the cells were fed every other day by removing all the medium and replacing it with fresh medium.
13. The amount of medium in a plate or flask was as follows:
    - 35 mm tissue culture dish, 2-3 ml
    - 100 mm tissue culture dish, 8-10 ml

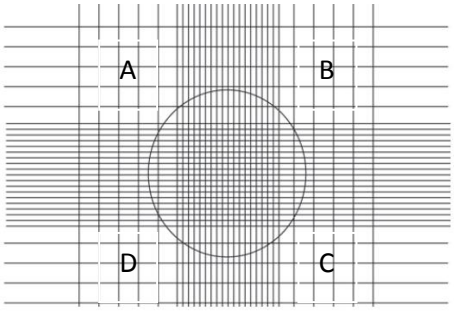
4.1.1.2. Feeding The Cells

1. The cells were fed every other day or every 3 days.
2. Half of the medium in the dish was aspirated off and was replaced with fresh medium.

4.1.1.3. Counting the cells

1. The cells were counted by trypan blue exclusion assay using a hemocytometer.
2. Formula to count number of cell:

$$\sum cell/ml = \frac{\sum \text{chamber A} + \sum chamber\ B + \sum chamber\ C + \sum chamber\ D}{4} \ x\ 10^4\ x\ dilution\ factor$$



4.1.2. MSC Injection

Single dose 5 x 106 MSC in 1 ml NaCl had been injected to the tail vein of SD rats after atherosclerotic induction occured. Subsequently, abdominal aorta was observed 3 weeks after this administration.

4.1.3. Mesenchymal Stem Cell Identification

4.1.3.1. Surface Marker Characterization of MSC

1. Micromass Cell Cultures

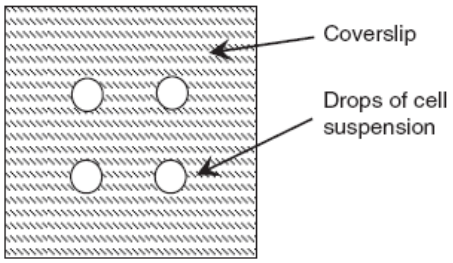
   Small cultures of MSCs grown on plain glass coverslips provided a convenient format for immunostaining.

   a. Glass coverslips (22 mm x 22 mm, thickness no. 1) were sterilized by separating them between layers of gauze in a glass 100 mm glass petri dish, lidded container and autoclaved.

   b. Placed one coverslip in each individual 35-mm Petri dishes.

   c. Trypsinized cells as described above, centrifuged, and resuspended at a concentration of 3000 cells per 10 ml in a complete growth medium.

   d. Using a micropipet, 2–4x 10 µl spots were pipeted on each coverslip. All spots on one coverslip were labeled with the same antibody.



e. Cover culture dish were placed in humidified 37 0C incubator and allowed cells to adhere for 45–60 min.

f. 2–3 ml complete growth medium were gently pipeted into each well, flooding the coverslips.

g. Cells were grown for a minimum of 2–3 days in complete growth medium to establish the micromass cultures before staining.

2. Fixation and Immunostainning

a. Coverslips were rinsed in culture dishes twice with PBS.

b. Cells were fixed for 30 min at ambient temperature with 10% buffered formalin, using ~1–2 ml formalin per dish.

c. Coverslips were washed twice with PBS.

d. Permeabilized cell membranes and blocked nonspecific binding by incubating fixed micromass cultures in blocking buffer (0.2% Triton X-100/0.5% bovine serum albumin in PBS) for 40 min, using ~1 ml per dish.

e. Antibodies were diluted in blocking buffer according to manufacturer's recommendations. Final concentrations in the range of 10–20 mg/ml for unlabelled primary mouse monoclonal antibodies and 4 mg/ml for

fluorescently labeled polyclonal secondary antibodies. Fifty microliters each of the primary and secondary antibody dilutions were required for each coverslip.

f. 50 µl of diluted primary antibody were pipeted onto a piece of Parafilm®. One coverslip were removed from the blocking buffer, drained briefly, and inverted on top of the drop of antibody solution. Incubated for 2 h, then returned coverslips to culture dishes and wash three times for 5 min in PBS.

g. Repeated using a new piece of Parafilm® and 50 µl of diluted secondary antibody for each coverslip. Incubated for 1 h, protected from light, then washed three times for 5 min in PBS.

h. DAPI (40 – 6 – diamidino – 2 - phenylindole, dihydrochloride) was used to label nuclei and actin filaments, respectively, to aid in visualizing the cells. Three-color images showing cells with stained nuclei, cytoskeleton, and surface markers were obtained with appropriate fluors.

i. After the final PBS wash, coverslips were drained and mounted on glass slides, inverted in 1–2 drops Prolong

Gold®, with or without DAPI then they were dried several hours or overnight before viewing.

4.1.3.2. Osteoblas differentiation

1. Material

   - Confluent cell
   - Osteoblast differentiation media

   Formula to make 100 mlosteoblas differentiation media

   - DMEM 89.0 ml
   - FBS 10 ml
   - 50 µM ascorbic acid 0.1 ml
   - 1 µM dexamethasone 0.1 ml
   - 10 mM β-glycerophosphate 1 ml
   - Penstrep 1 ml
   - Fungizone 0.5 ml

2. Procedure

   - Culture media were aspirated and changed with osteogenic differentiation media
   - Media were changed every 2-3 days by changing half media with new fresh osteogenic differentiation media.

3. Alizarine Red Staining

- Material
    - Confluent cell that cultured by osteogenic differentiation media
    - Alizarin red S solution: dissolve 2 gram alizarin red S in 100 ml deionized water. Filter, adjust pH to 4.1 – 4.3 with 1N ammonium hydroxide
- Protocol
    - Aspirate culture media and wash with PBS. *Caution:* confluent monolayers and calcium deposit are easily dislodge.
    - Fix for 15-30 minute in buffer formalin, then rinse 2 x with deionized water.
    - Add 2% alizarine red S. Stain for 3 min.
    - Wash very gently with water until excess stain is removed, about 3-4 washes.
    - View stained dish as soon as possible. Under phase microscopy, alizarin red stained calsium deposits appear as irregular red-orange crystals.

4.1.4. Animal Termination and Sample Collection

4.1.4.1. Tools and Materials

1. Wax board
2. Needle
3. Scalpel
4. Pincet
5. Scissor
6. Alcohol sprayer

4.1.4.2. Procedure

1. Kill animal by cervical dislocation
2. Put animal in wax board, clean rat abdominal hair, and spray abdominal by alcohol.
3. Laparatomy, and find abdominal aorta.

4.1.5. Tissue Processing

4.1.5.1. Tools and Materials

1. Bottles
2. Pincet
3. Buffer formalin 10%
4. Alcohol 30%
5. Alcohol 50%
6. Alcohol 70%
7. Alcohol 80%

8. Alcohol 90%
9. Absolute alcohol
10. Xylol
11. Paraffin
12. Poly-l-lysin
13. Canada balsam (etelan)

4.1.5.2. Procedure

1. Put abdominal aorta to the bottle containing buffer formalin 10% for fixation.
2. Tissue dehydration by putting abdominal aorta to alcohol high rise.
3. Tissue clearing by putting abdominal aorta to xylol.
4. Tissue impregnation
5. Tissue embedding

4.1.6. Immunostaining

4.1.6.1. Tools and Materials

1. Microtome
2. Object glass
3. Cover glass
4. Staining jar
5. Incubator
6. Becker glass

7. Paraffin block
8. Pipette
9. Stopwatch
10. Shaker
11. Microwave
12. Xylol
13. Alcohol
14. Water
15. H2O2
16. PBS
17. Blocking serum
18. Primer antibody (antibody TGF, antibody IL-1α, , antibody IL-6)
19. Secondary antibody
20. Streptavidin
21. Peroxidase-hydrogen peroxide-diaminobenzidine (DAB)
22. Hematoksilin.
23. Microscope

4.1.6.2. Abdominal Aorta IL-1α expression Immunohistochemistry Procedure

Paraffin sections (4 µm) of aortic arch were analysed by immunohistochemistry using the high-sheep IgG peroxidase

(SABC) kit. Aortic arch sections were incubated with sheep anti-rat IL-1α for 1 h at room temperature, followed by incubation with biotinylated anti-sheep secondary antibody for 1 h at room temperature. Sections were stained with diaminobenzidine and nuclei were counterstained with haematoxylin QS. Isotype-matched negative controls for IL-1α staining was also performed in atherosclerotic rat aortic arch in the absence of anti-rat IL-1α. Images were taken using a microscope and IL-1α positive spots were picked up and calculated.

4.1.6.3. Abdominal Aorta IL-6 expression Immunohistochemistry Procedure

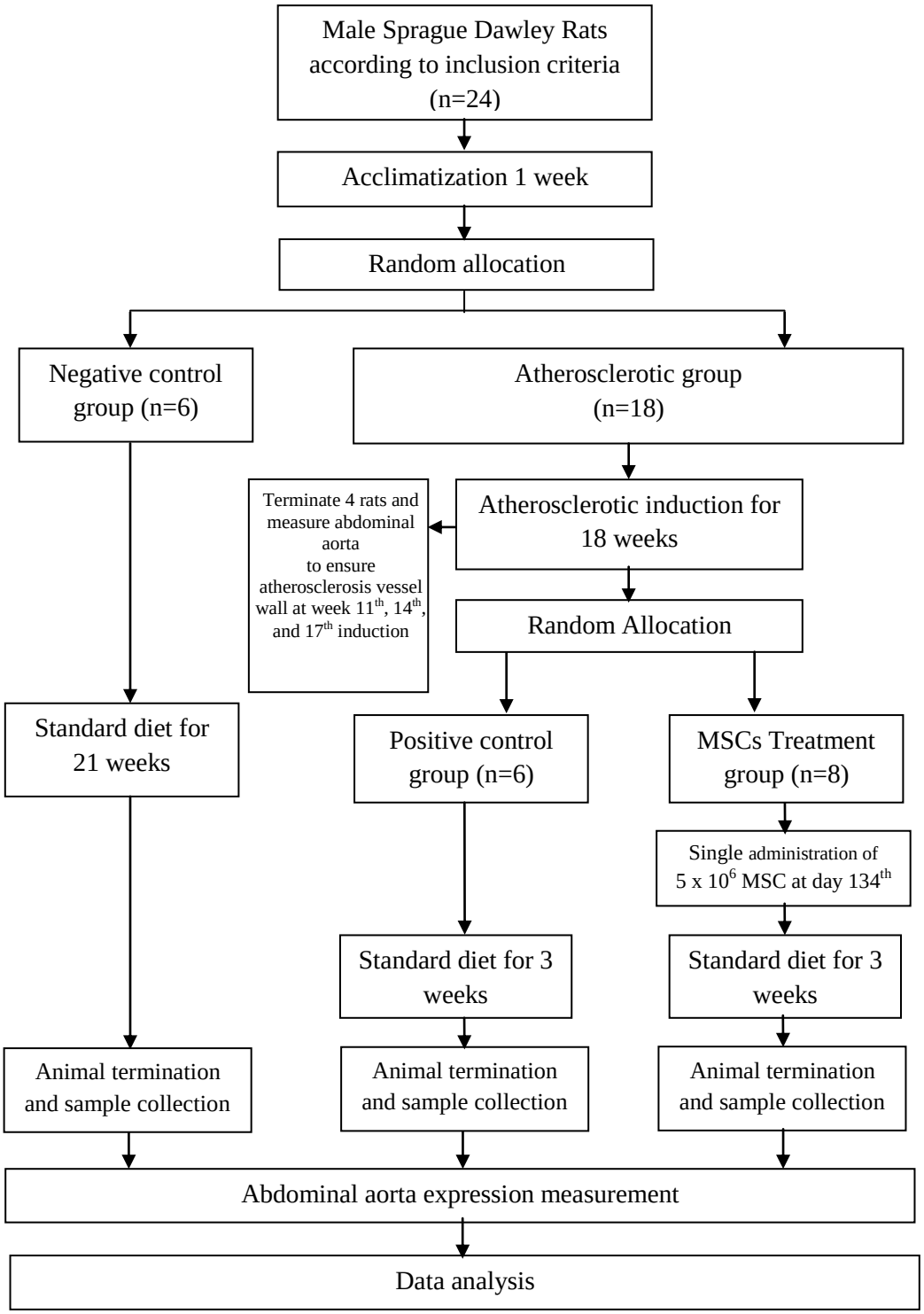
Paraffin sections (4 μm) of aortic arch were analysed by immunohistochemistry using the high-sheep IgG peroxidase (SABC) kit. Aortic arch sections were incubated with sheep anti-rat IL-6 for 1 h at room temperature, followed by incubation with biotinylated anti-sheep secondary antibody for 1 h at room temperature. Sections were stained with diaminobenzidine and nuclei were counterstained with haematoxylin QS. Isotype-matched negative controls for IL-6 staining was also performed in atherosclerotic rat aortic arch in the absence of anti-rat IL-6. Images were taken using a

microscope and IL-6 positive spots were picked up and calculated.

4.1.6.4. Abdominal Aorta TGF-β1 expression Immunohistochemistry Procedure

Paraffin sections (4 μm) of aortic arch were analysed by immunohistochemistry using the high-sheep IgG peroxidase (SABC) kit. Aortic arch sections were incubated with sheep anti-rat TGF-β1 for 1 h at room temperature, followed by incubation with biotinylated anti-sheep secondary antibody for 1 h at room temperature. Sections were stained with diaminobenzidine and nuclei were counterstained with haematoxylin QS. Isotype-matched negative controls for TGF-β1 staining was also performed in atherosclerotic rat aortic arch in the absence of anti-rat TGF-β1. Images were taken using a microscope and TGF-β1 positive spots were picked up and calculated.

4.2. Flow Chart of Study



4.3. Research Place and Time

4.3.1. Research Place

- Caring and treatment of the rats was conducted in Research Animal Facility of Pharmacology and Therapy Department, Faculty of Medicine, Gadjah Mada University.
- MSC was isolated and cultured in Physiology Laboratory, Faculty of Medicine, Gadjah Mada University.
- Surface marker identification of MSC was conducted in Physiology Laboratory of Medical Faculty, Gadjah Mada University and Pathology Anatomy Laboratory Sardjito Hospital.
- Tissue processing, and HE stainning were conducted in Pathology Anatomy Laboratory, Faculty of Medicine, Gadjah Mada University.
- Immunostaining and measurement of abdominal aorta were conducted in Pathology Laboratory of Sardjito Hospital.

4.3.2. Research Time

This Research was conducted at Gadjah Mada University for 6 months, from July 2013 to December 2013.

4.4. Data Processing and Analysis

The data on the atherosclerotic plaque event, IL-1α, IL-6 and TGF-β1 were *editied, coded* and *entered* process using SPSS 20 program, furthermore they have been *cleaned* and *organized* to data analysis preparation.

4.4.1. Descriptive Analysis

Descriptive analysis resulted in mean and deviation standard from atherosclerotic plaque event, IL-1α, IL-6 and TGF-β1 from each group. The result was presented in box plot graphics.

4.4.2. Inferential Analysis

Atherosclerotic plaque event, IL-1α, IL-6 and TGF-β1 were analyzed by nonparametric statistic using Kruskal-Wallis test to compare differentiation all three groups followed by wilcoxon-mann whitney test to compare differentiation each two groups.

4.5. Ethical Clearance

This study was approved by the Ethical Committee of Medical Research of Diponegoro University, No.383/EC/FK/RSDK/2013. This research has a purpose to investigate whether mesenchymal stem cells administration ameliorate atherosclerosis plaque in SD rats.