Cultivation of adipose-derived mesenchymal stem cells from Rat *Rattus norvegicus* by using mechanical + enzymatic method

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**Abstract.** Stem cells (stem cells) are the cells that do not have specific functions, can also differentiate into more than one type of cell that build tissues or organs of the body. In order to cultivate the stem cells from the primary tissue properly, it is necessary to optimize stem cells based on previous research methods. The design of this study was to cultivate mesenchymal stem cells from adipose (fat) tissue of Rat *Rattus norvegicus* by using mechanical + enzymatic method. The qualitative parameters that discussed were the option of handling isolation, time of incubation before cells reached confluence, and the morphology of mesenchymal stem cells obtained in each culture based on visual data using an inverted microscope. The quantitative parameters that analyzed were trypan blue assay and MTT assay. The results showed that the cultivation of mesenchymal stem cells were higher in the mechanical + enzymatic method compared with enzymatic methods. This method had a growth time from day 1 until it reached 80% confluence until day 7 and could be maintained until reached 90% confluence on day 9. Proliferation test by MTT assay showed the average of absorbance value was 0.942 with the cell number was $7.25 \times 10^4$ cells/µL. Further research is needed by flowcytometry assay and differentiation of this mesenchymal stem cells into osteocytes, adipocytes and chondrocytes.

1. Introduction

Mesenchymal Stem Cell (MSC) or also called multipotent stromal cell, is one of the stem cells in adult tissue that has the ability to self-renewal and differentiate into various types of cells, those are osteoblasts, chondrocytes, and adipocytes, myocytes, tenocytes and even neuron cells [1]. This non-hematopoietic cell have good capacity of proliferation and this is one of object that involved in tissue-injury and cellular-based therapy development for many diseases [2].

Mesenchymal stem cells, both in humans and mice, have been known that it could be isolated from various tissues, including adipose (fat), bone marrow, gingiva (periodontal), muscles, and bones [3]. For example, stem cells from adipose tissue or fat tissue, which is the most abundant tissue in the entire body of mammalian organisms [4]. This tissues is located in the subcutaneous (under the skin) and intraperitoneal (around the organ) and it composed of two form, namely white adipose tissue and
brown adipose tissue. Understanding of the biology of adipose-derived mesenchymal stem cells is very important in the development of cell-based clinical therapy [5].

The cultivation procedure in obtaining mesenchymal stem cells is generally same between species. Both humans and animals, the procedure for cultivate mesenchymal stem cells starts from tissue digestion through collagenase enzymes, then retrieves cell suspension from the supernatant, then performs density gradient centrifugation to obtain mononuclear cell fractions. The cells are then cultured on a dish or flask containing a medium. The stem cells obtained subsequently proliferate and eventually reach the confluence point. In rodents such as rat, cultivation procedures can be modified consider of tissues size and tissue of rats taken [6].

One of the main obstacles that facing researchers relating to stem cell cultivation is designing strategies to isolate and purify mesenchymal stem cells from components of mammalian adipose tissues, including rat adipose. The isolation and characterization methods of these mesenchymal stem cells are faced with a small number of stem cells in the tissue, but also the difficulty of determining differences in surface marker molecules in mesenchymal stem cells isolated in each tissue or organ [7]. This study was conducted to obtain the optimal method of cultivating mesenchymal stem cells found in rat adipose tissue and then analyzed quantitatively using the trypan blue test and MTT test.

2. Materials and Methods

2.1. Materials

The materials that used in this study are Biological Safety Cabinet Class II (BSC 2) [ESCO], CO2 incubator [ESCO], inverted microscope [Leica Mycrosystem], autoclave [Hirayama], centrifuge [Thermo Scientific], vortex [Thermo Scientific], waterbath [Memmert], 100 µm [BD Falcon] cell filter, T25 flask, petri dish, plate 96 well, 10-12 week male Rat Rattus norvegicus strain Sprague-Dawley strain, DMEM/LG (Low Glucose) [Sigma Aldrich], Ketamine, Phosphate Buffer Saline (PBS), Fetal Bovine Serum (FBS), trypsin [Gibco], penicillin-streptomycin (penstrep) [Gibco], collagenase enzyme type IA [Gibco], DMSO (dimethyl sulfoxide) [Merck], trypan blue [Gibco], MTT reagent [Invitrogen].

2.2. Methods

2.2.1. Collecting of the Rat Adipose Tissues. Mice surgery was conducted by anesthesia. Ketamine solution was injected through rat intravenous and then left for 5-10 minutes until the rat become limp. Rats were put into the dissection table then the ventral section opened using surgical scissors and anatomy tweezer. The intraperitoneal fat tissues of rat were taken around the intestine, stomach, pancreas and liver, while subcutaneous fat tissue were taken under the skin of the dermis. Fats were put into a beaker glass transport medium.

2.2.2. Isolation of Mesenchymal Stem Cells from Rat Adipose Tissue. Fat tissues were minced using a scalpel in a petri dish containing 2 ml of 0.075% collagenase type I solution. Tissues and collagenase solution were put together into a 15 mL tube then incubated in a waterbath at 37 °C for 30 minutes and vortexed with a 10 minute interval of incubation time. Collagenase was inactivated with 2 mL complete medium then incubated for 5 minutes at room temperature. Centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended with 2 mL DMEM/LG complete medium (supplemented with 10% FBS and 1,5% penicillin-streptomycin). Centrifuged again at the same speed. The supernatant was removed and the pellet was resuspended again with 3 mL DMEM/LG complete medium. The suspension was vortexed at a speed of 1200 rpm for 1 minute and filtered with a 100 µm cell strainer into the centrifuge tube. 1 mL of the suspended cells was culture into t25 flask which contained 3 mL of complete medium. Cells in the flask were incubated into 5% CO2 incubators at 37° C until it reach 70-80% confluence. Medium were changed every 3-4 days.

2.2.3. Morphological Observation of Rat Mesenchymal Stem Cells. Morphological observation and proliferation of mesenchymal stem cells from rat using an inverted microscope were carried out. Cell
culture images were taken every day with 10x and 40x lens magnifications then it collected as observation data.

2.2.4. Trypan Blue Assay (Hemocytometer). 1 µL of suspended cells were diluted into trypan blue solution with the ratio of 1: 1. The suspension then putted into the hemocytometer then cells were counted in the hemocytometer chamber number 1, 3, 7 and 9 on the microscope. The calculation results then put into the formula:

\[
\frac{\text{Number of Cells}}{\text{Number of Chambers}} \times \text{Dilution Factor} \times 10^4
\]

2.2.5. MTT Assay. Plate 96 well was filled with three variables: (1) blank (PBS 1x), (2) negative control (medium) and (3) positive control (medium + cells) with three times repetition. 100µL MTT (5 mg/mL) reagent was added to the plate containing negative control and positive control. Well then incubated into incubator of 37 °C, 5% CO₂ for 4 hours. 100% µL of trypsin was added to all plates and wrapped in aluminum foil. The plates are incubated for 24 hours in dark room at room temperature. Absorbance was measured using ELISA Reader at a wavelength 570 nm.

3. Results and Discussion

3.1. Results

3.1.1. Observation results of Rat Mesenchymal Stem Cells Isolates. The method that used was the mechanical and enzymatic method by collecting fat tissue of rat, then incubated together with the collagenase enzyme for 30 minutes, then centrifuged at a speed of 1200 rpm for 10 minutes, then resuspended with 2 mL of complete medium. Cells then culture into the flask and observed every day using an inverted microscope. The results of observations of rat mesenchymal stem cell isolates can be seen in Figure 1.
3.1.2 Trypan Blue Assay Results

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\text{Number of Viable Cells/µL} = \frac{\text{Number of Counted Cells}}{\text{Number of Chambers}} \times \text{Dilution Factor} \times 10^4
\]

\[
= \frac{145}{4} \times 2 \times 10^4
\]

\[
= 7.25 \times 10^4 \text{ sel/µL}
\]

3.1.3 MTT Assay Results. MTT assay was conducted to determine the proliferation of rat mesenchymal stem cells. PBS (1x) as blank, DMEM/LG complete medium as negative controls and complete medium + cells as positive controls. The absorbance value was measured using ELISA reader. The results of absorbance value of the MTT assay can be seen in Table 1.
### Table 1. Absorbance Value of MTT assay

| Variable          | Sample Repetition | Mean       |
|-------------------|-------------------|------------|
|                   | 1                 | 2          | 3        |          |
| Blank             | 0.033             | 0.033      | 0.035    | 0.033667 |
| Medium            | 0.196             | 0.154      | 0.143    | 0.164333 |
| Medium + Cells    | 1.109             | 0.869      | 0.849    | 0.942333 |

#### Figure 2. Average of Absorbance Value on MTT Assay

### 3.2. Discussion

The protocol used in this study was adapted from Zhu et al. (2013) [8] with relatively small modification. This method has succeeded isolated mesenchymal stem cells originating from adipose tissue in rat. The isolation results that obtained were then discussed based on the treatment at the isolation step, the incubation time to reach confluence, and the morphology of the mesenchymal stem cells obtained in each culture.

The results of the mechanical and enzymatic methods showed that on day 0 after the isolation, both in rats 1 or 2, stromal vascular fraction cells appeared to be spread fairly evenly in the flask culture. At the same time, the residual of rat adipose tissue that are seen in culture are also relatively large. This is due to the tissue digestion process through type I collagenase enzymes and mechanical process through mince the adipose tissues that carried out simultaneously at the time of isolation so the debris or residual tissue that produced was large enough.

The same results are shown by Alstrup et al. (2018) [9] study which isolated mesenchymal stem cells from human and pig adipose tissue by using mechanical methods through surgery and mince the tissues, then using enzymatic digestion by collagenase type IV showed better results than using only enzymatic digestion. According to him, due to the isolation using a mechanical method, it could separate the adipose tissue in a smaller size so that it increases the surface area of the tissue which could facilitate the collagenase enzyme to work optimally. The greater the surface area, the substrate molecules in the form of adipose tissue will receive the enzyme in a better concentration.

In isolation that has been done, it was seen that the fibroblast shaped morphology of the mesenchymal stem cells appeared to be quite widely spread by attaching to the flask on day 1 to day 3, but in small numbers and relatively small sizes. This cultivation method, could be maintained fibroblast form of mesenchymal stem cell starting from day 1 to day 9 after isolation.
On the 4th day until the next day, the colonies of the mesenchymal stem cells at the bottom of the flask appeared to be enlarged and clear. Forms of fibroblasts in mesenchymal stem cell colonies could be maintained until the culture reached at the confluence point on days 8 to 9 after isolation. Mesenchymal stem cell colonies were seen reaching 90% confluence on day 9 which indicated the cells at the highest confluence point so that subcultures should be needed.

Volovitz et al. (2016) [10] suggest that cell suspension with a number of lower separated tissues generally produced fewer cells, but not vice versa. Suspensions with a higher number of separated tissues even make the cells turn to death. The reason for this that perhaps the quality of mesenchymal stem cells thought to be lower if the incubation time of enzymatic digestion longer so that the number of separated tissues became higher.

4. Conclusion
Based on the results obtained through a visual observation process using an inverted microscope, the results of optimal mesenchymal stem cell cultivation on the mechanical + enzymatic method with a growth time of 1 to 7 days until it reached a confluence of 80% and were maintained to reach a 90% confluence at 9th day. The proliferation test with MTT assay obtained an average of absorbance value was 0.942 with a cell number was 7.25 × 10⁴ cells / µL.

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