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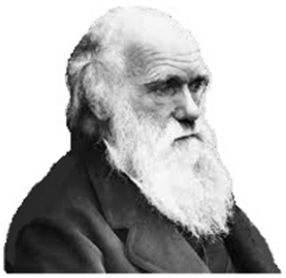

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Seeding cell number required for optimal lipid accumulation during adipocyte differentiation using 3T3-L1 cell line

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ABSTRACT

Obesity is one of the major causes of metabolic diseases such as diabetes and heart attack, and, hence, can lead to low quality of life**.** Elaborating adipocyte differentiation is very crucial for formulating the treatment and prevention of obesity. The objective of this study is to investigate the seeding cell number required to obtain optimum lipid accumulation during adipocyte differentiation using the 3T3-L1 cell line. Two sets of 5.48×10^4 (for Day 0 and Day 8 of differentiation), of 10.96×10^4 (for Day 8) and of 21.92×10^4 (for Day 8) of 3T3-L1 cells were seeded in each wells of a 12-well plate. Isobuthylmethylxanthine (IBMX), Dexamethasone, and Insulin-containing differentiation cocktails was added into the medium at Day 0 for 48 hours. The medium was changed every two days. Day 0 and Day 8 samples were then stained using Oil Red O and were examined under the microscope to observe the lipid droplets (red-coloured). The lipid droplets were quantified by measuring the absorbance at

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wavelength of 550 nm. In the study, seeding the number of 10.96×10^4 cells produced very significantly higher lipid accumulation, as compared with seeding the number of 5.48×10^4 cells. However, doubling the seeding number into 21.92×10^4 cells did not increase the lipid droplets significantly. This study found that the optimum seeding number to obtain the maximum lipid droplets during 3T3-L1 adipocyte differentiation was 10.96×10^4 cells.

***Keywords*:** 3T3-L1 preadipocytes, differentiation, lipid accumulation, seeding cell number

1. INTRODUCTION

The prevalence of obesity is still high worldwide. The World Health Organization (WHO) reported that global obesity has nearly tripled since the year 1975.[4] The prevalence of overweight and obesity in South East Asia is also now increasing, ranging from 8% to 30% in adult males and 8 to 52% in adult females.[1] The prevalence of obesity in adults in Indonesia was 14.6%.[1] The prevalence of obesity in West Java province was 10 to 19 %.[3,4] The region with the highest prevalence of obesity in Indonesia was the eastern region.[2] Adipocyte differentiation or adipogenesis plays important role in obese patients. Adipogenesis is increased in an obese person which leads to their obesity.[14] Adipogenesis can occur due to many reasons but very rarely due to genetical alteration.[9,10] Increased adipocyte differentiation occurs in people who undergo a sedentary lifestyle and people who practices a poor diet consisting of high fat foods.[9]

Since obesity can also be said as excess of lipid accumulation in adipocytes, finding out a way to reduce adipocyte formation might give a chance to formulate treatment and prevention of obesity. For that purpose, 3T3-L1 preadipocyte cell lines, which were originally cloned from 3T3 mouse embryo fibroblasts,[12] were used to study the differentiation of the preadipocyte cells to adipocytes in-vitro under chemical stimulation, also known as differentiation cocktail.[3,8]

Although numerous studies regarding adipocyte differentiation have been performed worldwide, the protocol may vary in different regions and with different origin of cell lines.[5,13] Optimized protocol is required to get the optimum number of differentiated adipocytes. It is very important because in further studies researchers can take advantage of the optimized protocol to identify substance(s) which has/have inhibition effect on adipocyte differentiation, suggesting potential antiobesity effects.[6]

The purpose of this study is to investigate the suitable optimum seeding cell number required to obtain maximum lipid accumulation during adipocyte differentiation using 3T3-L1 preadipocyte cells at Cell Culture and Cytogenetic Laboratory, Advanced Biomedical Laboratory, Universitas Padjadjaran (UNPAD) located in UNPAD Teaching Hospital, Jalan Eijkman No.38, Bandung, West Java, Indonesia.

2. MATERIALS AND METHODS

2. 1. Research Design

This is a quantitative analytic study. The purpose of this research is to find the optimum seeding cell number in adipocyte differentiation to develop a protocol for the differentiation of 3T3-L1 preadipocyte cells into adipocytes.

2. 2. Cell Differentiation

In conducting this study, we took advantage of 3T3-L1 preadipocyte cell line. Three different mediums were used for the process of cell differentiation: MDI (IBMX-dexamethasone-insulin)-containing DMEM-10% FBS-P/S (penicillin-streptomycin) which was called as induction medium, 167 nM Insulin containing DMEM-10% FBS-P/S, and empty DMEM-10% FBS-P/S medium.

The MDI induction medium consists of DMEM-10% FBS-P/S 100 ml, 0.5 mM IBMX, 0.25 µM dexamethasone, and 1 µg/ml insulin. MDI medium should be stored at a temperature of 4 degree Celsius and should be protected from light. Twelve-well plates were used in all experiments in this study.

On day -5, cell passaging and cell counting should be done (processes explained below) and 2 sets of 5.48×10^4, 10.96×10^4, 21.92×10^4 cells are seeded in each wells of 12-well plate. DMEM-10% FBS-P/S is added to the wells. On day -2, the medium is changed with same medium (DMEM-10% FBS-P/S). On day 0, For Day 8 samples, medium is replaced with MDI medium. For day 0 samples, the cells are fixed using 4 % formaldehyde solution and stored at 4 degree Celsius.

The other set of cells (Day 8 samples) are left to differentiate in the MDI medium for 48 hours. On day 2, the medium of Day 8 samples is replaced with DMEM-10% FBS-P/S-167 nM Insulin and left to differentiate for 48 hours. On day 4, the medium of Day 8 samples is replaced with DMEM-10% FBS-P/S medium and left to differentiate for 48 hours. On day 6, the medium of day 8 samples is replaced with new DMEM-10% FBS-P/S medium and left to differentiate for 48 hours. On day 8, the cells are now fully differentiated and ready to be stained to determine the percentage of lipid accumulation. The cells (Day 8 samples) as well as Day 0 samples (which was already fixed and stored at 4 degree Celsius) are stained with oil red O (explained below). Then the samples are placed in a microplate reader for the number of lipid cells to be counted the lipid accumulation of Day 0 and Day 8 samples were compared.

2. 3. Cell Counting using Improved Neubauer method

Hemocytometer was used to count the cells.[15] Trypan blue dye was added to the cells to identify live cells.

2. 4. Oil Red O Staining

The cells were fixed with 4% formaldehyde before the staining. After being washed with 60% isopropanol, the cells were stained with Oil Red O solution for 15 minutes. The cells were then rinsed with 60% isopropanol again. Lipid accumulation was stained red.

2. 5. Oil Red O Quantification

Isopropanol was added to the wells to elute the red stain. The eluant was then transferred to another 96 well plate and the absorbance was measured at wavelength of 550 nm. Compare the absorbance value between the wells containing different samples.

2. 6. Statistical analysis

The data was presented in mean$\pm$SD and analyzed using Microsoft Excel 2016.

3. RESULTS

3. 1. Qualitative analysis of lipid accumulation

The macroscopic pictures showed that the well plate in day 0 containing 5.48×10^4 number of cells had no lipid accumulation, confirming that they were still preadipocytes. Day 8 samples containing 10.96×10^4 and 21.92×10^4 number of cells had more abundant lipid accumulation, suggesting mature adipocytes, whereas day 8 sample containing 5.48×10^4 number of cells had less lipid accumulation (Figure 1).

Lipid accumulation in sample with seeding number of 10.96×10^4 cells was very significantly higher as compared to sample with seeding number of 5.48×10^4 cells, while the difference between day 8 sample of 10.96×10^4 cells and 21.92×10^4 cells was not very significant. The microscopic pictures with 40x and 100x magnification support the results from the macroscopic pictures (Figure 1).

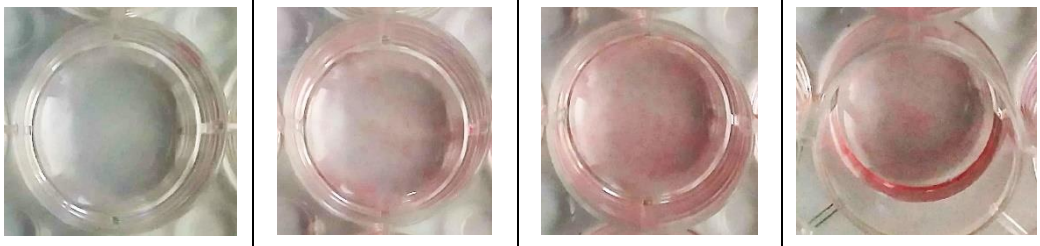
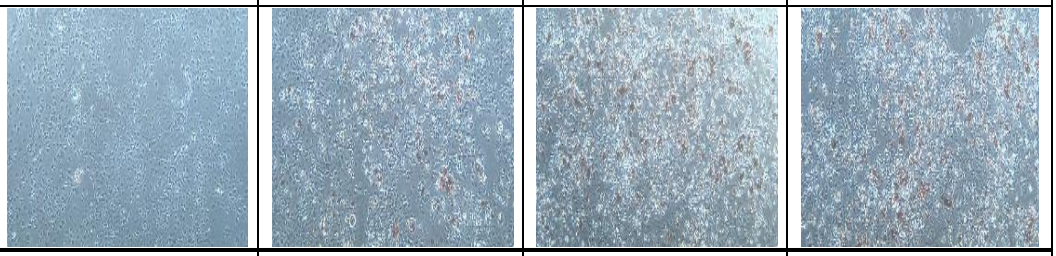
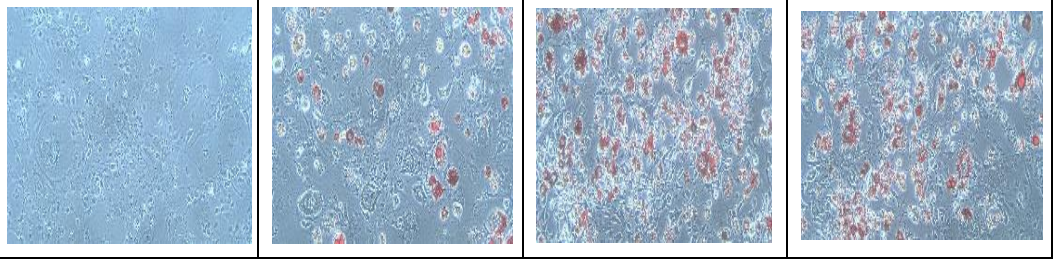
	Day 0		**Day 8**	
Seeding cell number	5.48×10^4	5.48×10^4	10.96×10^4	21.92×10^4
Macroscopic				
Microscopic 40 × magnification				
Microscopic 100 × magnification				

Figure 1. Macroscopic and microscopic pictures of cells after oil red O staining. The cells were seeded in three different numbers (as indicated in the figure) on Day -5. MDI stimulation was started on Day 0. Oil red O staining was performed on Day 0 (before MDI stimulation) and eight days after differentiation (Day 8).

3. 2. Quantitative analysis of lipid accumulation

Figure 2 showed that Day 8 of sample with seeding number 10.96×10^4 cells produced the optimum number of lipid staining intensity at the wavelength of 550 nm and confirms that it had very significantly more lipid accumulation as compared to Day 8 of 5.48×10^4 cells seeding number. To double the seeding number from 10.96×10^4 cells into 21.92×10^4 cells did not increase the lipid accumulation very much (Figure 2).

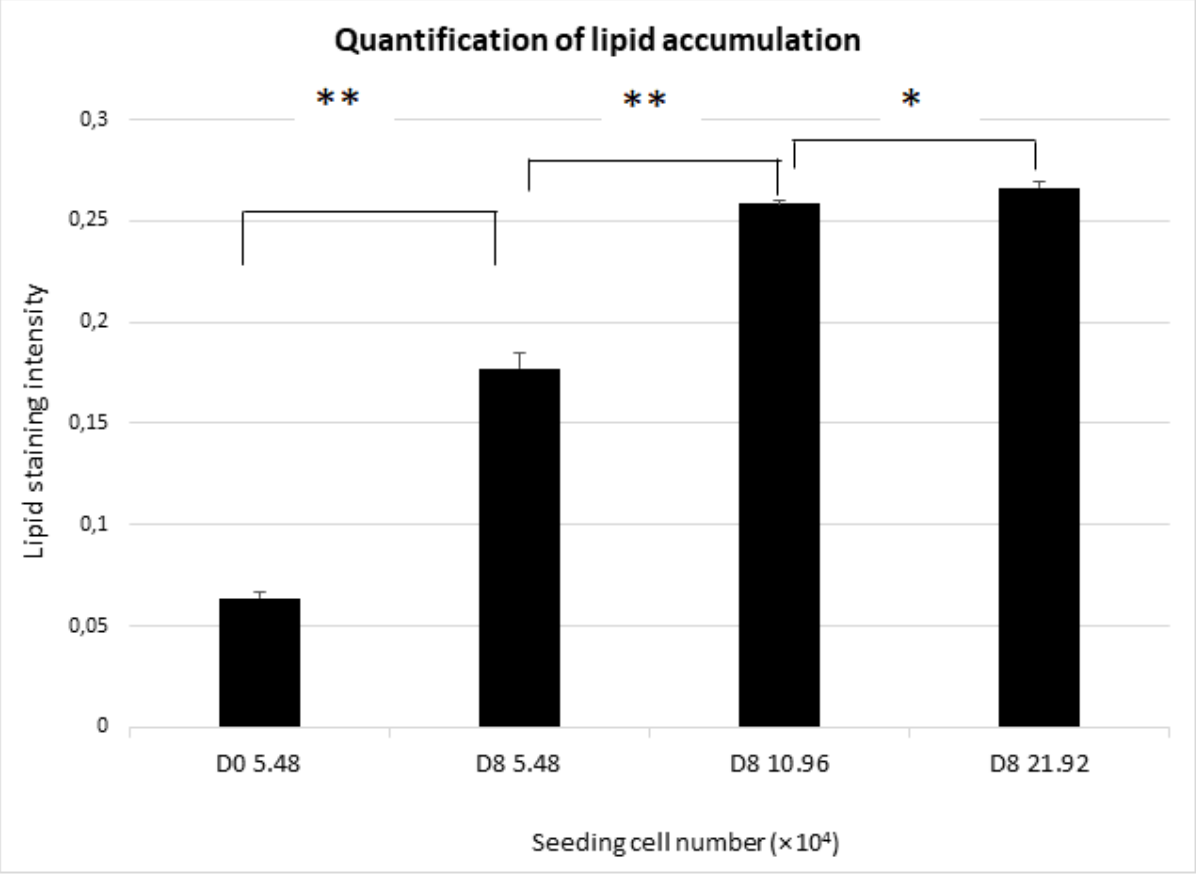


Figure 2. Quantification of lipid droplets. Oil Red O staining was dissolved in 2-propanol and the colour intensity was determined by measuring absorbance at wavelength of 550 nm. *p<0.05, **p<0.01. D0: Day 0, D8: Day 8.

4. DISCUSSION

The objective of this study was to investigate the optimum preadipocyte seeding number to acquire maximal lipid accumulation during adipocyte differentiation using 3T3-L1 cells. Our study suggested that seeding cell number of 10.96×10^4 produced the optimum lipid accumulation during 3T3-L1 differentiation.

Our study also implied that higher seeding number did not mean that it will result in higher lipid accumulation because a very high seeding cell number can also decrease the differentiation efficiency.[11] This is proved in our study when the difference between day 8

sample containing 5.48×10^4 seeding cell numbers and day 8 sample containing 10.96×10^4 seeding cell numbers were very significant but the difference between day 8 sample containing 10.96×10^4 seeding cell numbers and day 8 sample containing 21.92×10^4 seeding cell numbers was less significant. This might be explained by a study conducted by Zebisch *et al* suggesting that higher seeding cell numbers decreased the differentiation efficiency of the cells.[7]

Protocol of 3T3-L1 differentiation might vary among laboratories. Every cell culture laboratory has their own protocol which includes the seeding number, concentration of differentiation cocktail, etc. This study was conducted to find out the optimum seeding cell number for maximal lipid accumulation. This optimum seeding number of cells can be utilized for further study, for example, for identification of herbal extracts which can inhibit adipocyte differentiation.

5. CONCLUSION

The optimum seeding cell number for maximal lipid accumulation during differentiation of 3T3-L1 preadipocytes into mature adipocytes was 10.96×10^4 cells.

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