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The effect of cell suspension age on the result of simple spot method

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ABSTRACT

Aim: This study aimed to compare the quality of slides that are made from fresh suspension and those made the next day until some days afterward. **Experimental:** Slides were made in 5 replications directly after the cell suspension was ready, and the rest of the cell suspension was kept in the refrigerator. Further, slides were made on day 2, 3, 5 and 6 in 5 replications each. First the slides were checked for the presence of cells. The intact and damaged cells on each slide were counted, noted and tabulated. The percentage of intact cells per slide was calculated. **Results:** All the slides that were made directly and on day-2 contained cells, while after day-2, not all of the slides contained cells. Moreover, the slides that were made on day-6 only contained 2-4 cells per slide. The mean of the percentage of intact cells from the slides that were made directly, on day-2, -3, -5, and -6 were 65, 52, 29, 0, and 67% respectively. **Conclusion:** Cell suspension age has an impact on the success rate of cell containing slides and to get enough intact cells, fresh cell suspension until at last day-2 should be used.

KEYWORDS

Intact cells;
Spot method;
Suspension age;
Culture;

INTRODUCTION

Stem cells are very promising for regenerative medicine. Immune-fluorescent staining and flow cytometry analysis was usually done to identify the stem cells[1-3] and a sample of 10^6 cells is usually needed for the analysis[2]. The use of so many cells for analysis is a waste. Therefore we have developed a simple spot method, which produce cell containing slides like those that were produced using a cytospin, but without using a cytospin apparatus, and thus it is called the simple spot method[4].

The simple spot method was developed to visualize and stain cultured cells or stem cells in suspension[4], which can be used concomitantly with immunohisto-chemical staining to identify the various kinds of stem cells. This method only

needs around several hundred of cells for one marker, and has been used to show morphological changes due to spontaneous differentiation of adipose tissue derived stem cells[4]. In the simple spot method, the slides were made on the same day after the cultured cells or tissues were subjected to enzymatic digestion[4]. Harvesting cultured cells or processing tissues to get cell suspension need time, and time constraint due to laboratory facility usage may lead to difficulties in managing to make the slides directly after harvesting. Further, doing research in a university, where educational tasks take part of researchers time, necessitates strict time management in doing research. Therefore, it will be advantageous if we can keep the cell suspension overnight or for several days and make the slide the next day or after some time when the time is available. However, we do not know whether the quality of the slides that are made from kept cell suspension will be as good as the slides that are made from fresh suspension. Therefore, this study aim to compare the quality of slides that are made from fresh suspension and those made the next day until some days afterward.

EXPERIMENTAL

This is a cross sectional comparative study that is part of a research on adipose tissue derived cells, which has got an approval from the ethical committee of the Faculty of Medicine University of Indonesia. This research was done in the Immunology and Endocrinology Integrated Laboratory, and the Department of Histology, Faculty of Medicine, University of Indonesia, from March to May 2010.

Sample

Adipose tissue (1cm x1cm x2 cm) was obtained from a patient that was agree to donate the tissue and had signed the informed consent form.

Tissue processing

Tissue processing was done according to a modification of the method of Astori et al[1], and Jurgens et al[5]. In brief, the tissue was minced and subjected to digestion by 0.075% collagenase-type1 (Gibco) in phosphate buffered saline (PBS) pH 7.4 at 37^{o}C for 60 minutes with intermittent shaking every 5 minutes. The infranatant was centrifuged at 800g for 10 minutes and the pellet was suspended in culture medium containing 10% fetal bovine serum (FBS, Biowest), 1% Penicillin - Streptomycin (Lonza) and 1% Amphotericin B (Biowest) in Dulbeco Minimal Eagle Medium (DMEM, Lonza). The cell suspension was used to make the spot specimen slides as were done before[4,6].

Slide preparation

On each slide, 4 spots of 1µl cell suspension were applied using a micropipette and yellow tip. Slides were made in 5 replications directly after the cell suspension was ready, and the rest of the cell suspension was kept in the refrigerator at 4^{o}C. Further, slides were made on day 2, 3, 5 and 6 in 5 replications each. Before taking the cell suspension and making the slides, the cell suspension was homogenized.

All slides were fixed directly after the spotted specimens dried, and stained by hematoxylin eosin. Fixation was done using 95% alcohol for 5 minutes[4, 6].

Data collection and analysis

The specimens were numbered and analyzed under the microscope blindly. First the slides were checked for the presence of cells. All the cells that were found in the spot specimens were examined and counted, and divided into 2 groups: intact cells and damaged cells. Intact cells should have a distinct nucleus and cell membrane, and damaged cells were either having a blurred appearance, damaged nucleus or cell membrane.

The number of intact and damaged cells was noted for each spotted specimens and tabulated, and the percentage of intact cells were calculated for each slide. The difference in the percentage of intact cells from direct specimen, day 2, 3, 5, and 6 is analyzed using ANOVA if the data is homogenous and normally distributed. In addition the means and

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standard deviation (SD) for the various suspension ages were computed. If the data does not fulfill the criteria for ANOVA, non parametric statistic will be done. When there was significant difference in intact cells between the various suspension ages, statistical analysis was followed by the LSD test to know the difference. All data analysis was done using SPSS 11.5 for Windows. The time when the specimen was made that yielded the highest number of intact cells was regarded as the best.

RESULTS

All the slides that were made directly and on day-2 contained cells, while after day-2, not all of the slides contained cells. However, only four out of the five slides made on day-3 contained cells, while only 3 out of the five slides made on day-5 and -6 contained cells. Moreover, the slides that were made on day-6 only contained a few (2-4) cells per slide. The mean and SD of the percentage of intact cells from direct specimen, day 2, 3, 5, and 6 can be seen in TABLE 1.

TABLE 1 : Percentage of intact cells at various cell suspension ages.

Spot specimens made	Mean	SD
Directly	64.60	23.12
On day 2	51.60	15.90
On day 3	28.75	14.45
On day 5	0	0
On day 6*	66.67	28.89

***Cell number only 2-4 cells/slide**

Statistical analysis showed that there was a significant difference between the five groups of cell suspension age, and the p value of LSD test can be seen in TABLE 2. Therefore, the best time to make the slides is directly after the cell suspension is ready, as the highest number of cell containing slide and the highest percentage of intact cells were achieved at that time.

TABLE 2 : P value of LSD test between the various suspension ages.

	Directly	Day-2	Day-3	Day-5	Day-6
Directly		.298	.013*	.000*	.884
Day-2	.298		.094	.002*	.296
Day-3	.013*	.094		.067	.020*
Day-5	.000*	.002*	.067		.001*
Day-6	.884	.296	.020*	.001*	

DISCUSSION

In this study, the percentage of intact cells in the specimens that were directly made after the cell suspension was ready showed that only 64.60% of the cells were intact. This result pointed out that our collagenase type 1 treatment was too long, the concentration was too high or the mixing was not gentle enough for our sample. Thus, if the cells were cultured they might not grow satisfactorily as many cells were damaged. However, another study that applied 1 hour digestion using higher concentration of collagenase type 1 (0.1%) showed that the cells grew satisfactorily, indicating that most of the cells should be intact[7]. In different laboratories, different tissue treatment procedures for adipose tissue to be cultured to yield stem cells were done, and they reported a successful culture result. Most of them used collagenase type1 as was use in this study, i.e. 0.075% for 30 minutes[3], or 45 minutes[1], or 0.1% for 1 hour[7], and another study used collagenase A 0.1% for 45 minutes[8]. From the various concentration and time of digestion used in different laboratories, we concluded that we should reduce the treatment time to get a better initial result.

In stem cells research, this spot method to make specimens from cell suspension, either from digested tissue or from trypsinized cell culture will be very valuable if this method can be used together with immunocytological staining. Immunocytochemical staining for the various stem cell markers may substitute cell counting using the high cost flow cytometer. Theoretically, various markers that can be used to detect and to measure the numbers of various kinds of stem cells such as CD 34 (hematopoetic and endothelial stem cell marker), and various mesenchymal stem cell markers such as CD 13[2], CD 29 and CD44[7], and

CD 73, CD 90 and 105[9], can be applied for the simple spot method. Therefore, it is very important to know, until how long the cell suspension can be kept, but still give a sufficient amount of intact cells to be analyzed.

Further, our result showed that cell suspension age had an influence on the number of cell containing slides, and the percentage of intact cells in the spotted specimens. The number of cell-containing slides tended to decrease with the increase in cell suspension age. This fact might be due to attachment of the cells to the plastics of the Eppendorf tube that was used to keep the cell suspension, as collagenase digested adipose tissue was proven to yield plastic adherent mesenchymal stem cells[1,3,5,7].

Overall, the older the age of cell suspension, the less was the percentage of intact cells, except the slides that were made on day-6 that showed the highest percentage of intact cells. However, those slides only contained 2-4 cells, which were not representative due to the very few cell number. Therefore, the cell suspension age that still can be used to make spot specimens with enough intact cell yields is until day-2.

In this study, we kept the cells at 4^{o}C to reduce the metabolic activity, to prevent the cells from mitosis and to avoid medium change, in order to get a consistent cell number, as mitosis and washing step before medium change will cause inconsistent cell number. However, as the sample was obtained from only one patient, further studies on more samples are needed to be certain that the result is consistent and there are no inter-individual differences in cellular integrity.

ABREVIATION

PBS= phosphate buffered saline
FBS= fetal bovine serum
DMEM= Dulbeco Minimal Eagle Medium
SD= standard deviation

CONCLUSION

Cell suspension age has an impact on the success rate of cell containing slides and to get enough intact cells, fresh cell suspension until at last day-2 should be used.

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