Effect of adipose tissue processing procedures in culture result: a study preliminary

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Abstrak

Latar belakang: *Ada berbagai cara pemrosesan jaringan lemak sebelum dikultur, tergantung jenis sampelnya yang dapat mempengaruhi hasil kultur. Penelitian ini bertujuan membandingkan berbagai modifikasi prosedur kultur dan subkultur jaringan lemak yang disesuaikan dengan kondisi lab yang ada.*

Metode: *Penelitian ini adalah penelitian deskriptif yang dilakukan di Makmal Terpadu Imunologi dan Endokrinologi, Universitas Indonesia, mulai Oktober 2009 sampai April 2010. Kami membandingan tiga cara pemrosesan, berbagai jumlah sel yang ditanam yang tergatung jumlah perolehan sel, dan dua cara subkultur, lalu membandingkan hasilnya dalam hal jumlah sel yang dihasilkan dan waktu yang diperlukan. Pada cara pemrosesan pertama, pencernaan dengan collagenase-1 dilakukan selama 30 menit dan jumlah sel yang ditanam adalah 24.000 dan 36.000 sel per wadah kultur; pada cara kedua, pencernaan dengan collagenase-1 dilakukan selama 60 menit dan jumlah sel yang ditanam adalah 24.000, 48.000, dan 72.000 per wadah kultur; dan pada cara ketiga, sisa jaringan lemak dari pemrosesan pertama dicerna kembali selama 45 menit dan jumlah sel yang ditanam adalah 74.000 dan 148.000 per wadah kultur. Perbedaan cara subkultur adalah pada ada atau tidaknya tahap pencucian.*

Hasil: *Prosedur -1 menghasilkan jumlah sel yang paling sedikit, dan sesudah dikultur, selnya tumbuh sangat lambat, dan terkontaminasi sebelum panenan kultur primer. Prosedur-2 dan -3 berhasil menumbuhkan kultur primer. Beberapa kultur terkontaminasi, sehingga tidak dapat dilanjutkan dengan subkultur, dan hanya satu cara pemrosesan (prosedur-2: pencernaan collagenase-1 selama 60 menit tanpa penggunaan dapar pelisis, dan jumlah sel yang ditanam 48.000 dan 72.000) yang berhasil menyelesaikan semua proses yang direncanakan sampai subkultur ketiga. Walaupun beberapa prosedur tidak mencapai subkultur ketiga, hasilnya tetap dapat disimpulkan.*

Kesimpulan: *Penelitian pendahuluan ini menunjukkan bahwa pencernaan collagenase-1 selama 60 menit dipadu dengan goyangan berkala setiap 5 menit dan jumlah sel yang ditanam sekitar 50.000 atau lebih, diikuti dengan cara subkultur tanpa tahap pencucian memberi hasil yang terbaik.* ***(Med J Indones 2011; 20:15-9)***

Abstract

Background: There are various methods of processing adipose tissue before culture, depending on the adipose tissue samples. The aim of this study is to compare several modifications of culturing and sub-culturing procedures of adipose tissue to fit the condition in our laboratory.

Method: This is a descriptive study that was done in the Immunology and Endocrinology Integrated Laboratory, University of Indonesia, from October 2009 to April 2010. Three adipose tissue processing procedures, various amount of seeding and two subculture methods were compared in term of cell yield and time needed. In the first procedure, collagenase-1 digestion was done in 30minutes, cell seeding were 24,000 and 36,000 per flask; in the second procedure, collagenase-1 digestion was done in 60minutes, cell seeding were 24,000, 48,000, and 72,000 per flask; and in the third procedure, the adipose tissue remnants from the first procedure were again digested for another 45 minutes, cell seeding were 74,000, and 148,000 per flask. Difference in subculture methods were the presence or absence of washing step.

Result: Procedure 1 yielded the lowest amount of cell, and after culture, the cells grew very slow, and was contaminated before harvest of primary culture. Procedure-2 and -3 succeeded to yield primary cultures. Some of the cultures were contaminated, so that further subculture was not applicable, and only one tissue processing procedure (procedure 2: 60 minute collagenase-1 digestion, without lysis buffer, cell seeding 48,000 and 72,000) could complete the three subcultures. Though some of the procedures could not be completed, final result could be concluded.

Conclusion: In this preliminary study, 60 minute colagenase-1 digestion with intermittent shaking every 5 minutes and cell seeding around 50,000 or more, followed by subculture method without washing step gave the best result. ***(Med J Indones 2011; 20:15-9)***

Key words: *collagenase-1, primary culture, subculture, stromal-vascular fraction*

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Adipose tissue stem cells are adult stem cells that have similar properties to the previously identified bone marrow mesenchymal stem cells. They can be isolated from adipose tissue stromal vascular fraction (SVF) that is called adipose stromal compartment.[1, 2] The stromal vascular fraction derived cells are also called processed lipo-aspirate (PLA) cells,[2] or alternatively, adipose tissue derived mesenchymal stem cells (AT-MSCs). Adipose tissue stem cells have various differentiation[2] and angiogenic potentials.[3] In addition, they have immuno-suppressive properties,[4, 5] and might be used to treat autoimmune diseases.[6] Moreover, the ease in collecting the samples compared to that of bone marrow make adipose tissue stem cells very promising for regenerative medicine.[7]

There are various methods of processing adipose tissue before culture, depending on the adipose tissue samples. Processing adipose tissue samples from lipoaspirate is different from resection derived samples. Further, there are various procedures for resection derived samples. The aim of this study is to compare several modifications of culturing and sub-culturing procedures for resection sample to fit the condition in our laboratory.

METHODS

This is a descriptive study that is part of a research on adipose tissue derived stem 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, Faculty of Medicine, University of Indonesia, from October 2009 to April 2010

Sample

The adipose tissue resection sample was taken by a plastic surgeon, after the patient who underwent a plastic surgery got information about the research and had signed the informed consent form.

Tissue processing

In this study, we compared several procedures of tissue processing. We also compared different amount of cells that were cultured. First of all, the adipose tissue sample was washed from contaminating blood in phosphate buffered saline (PBS) pH 7.4 until the washing solution was clear, cut into 2 pieces, weighted, and the weights were noted. All processing was done in aseptic condition using sterile instruments and solutions.

Both adipose tissue pieces were subjected to collagenase-1 digestion. Digestion was done in a 50mL tube containing 25mL 0.075% collagenase-1 in PBS pH 7.4 at 37^{o}C, and was gently shaken every 5 minutes.

In this preliminary study, we tested 3 kinds of modified procedures using the available equipments in our lab. In the first and second procedure, digestion was done in 30 and 60 minutes respectively. In the third procedure, the adipose tissue remnants from the first procedure were again digested for another 45 minutes.

After digestion, the floating adipose tissue was removed and the infranatant was placed in 15mL tubes, centrifuged at 800g, and the supernatant was discarded.

Culture and subculture

In the first and second procedure, the pellets were dissolved in 2.5 and 3 ml tissue culture (TC) medium respectively, and the cells were counted in Neubauer chamber.

In the third procedure, the pellet was dissolved in 4mL lysis buffer and incubated at 37^{o}C for 5 minutes, then centrifuged at 800g, and the supernatant was discarded. The pellet was dissolved in 3mL TC medium, and the cells were counted in Neubauer chamber.

The TC medium contained 10% fetal bovine serum (FBS, Biowest), 1% penicillin streptomycin (Lonza) and 1% Amphotericin B (Biowest) in Dulbeco Minimal Eagle Medium (DMEM, Lonza).

According to cell yield of every procedure, the cells were then seeded at different cell numbers (Table 1) in a final volume of 8mL TC medium in 25mL TC flask, and incubated at 37˚C with 5% CO2.The cultures were checked for the presence of contamination every day. Contaminated flasks were discarded. Beginning at day two, the flasks were checked every day for cell attachment. After the cell attachment of all flasks, the TC medium was replaced twice a week, every Monday and Thursday, or more frequent when the color changed into orange.

When the cells were 40 - 80% confluence, subculture was done. We tested two subculture procedures, i.e. with and without washing step (direct subculture). Subculture (until subculture-3) was done by replacing the TC medium by 1.5mL of 0.05% trypsin in PBS pH 7.4, until the cells were detached, and trypsin solution was added when the cells were not detached after 10 m minutes. Further the trypsin was neutralized by a same amount of TC medium. The number of cells in the cell

suspension was counted. In direct subculture, half of the cell suspension was directly sub-cultured, and in the procedure with washing step, the other half was washed by PBS pH 7.4 before sub-culturing. When the yield was abundant, part of the cell suspension was directly sub-cultured, and a same amount was washed before sub-cultured, and the rest was cryopreserved. For direct subculture, the TC medium was replaced the next day. Further, for both subculture procedures, the TC medium was replaced twice a week, or more frequent when the color changed into orange.

Data collection and interpretation

The total numbers of cell yield per gram of adipose tissue for every procedure were noted. Further, the days needed until the cells attached and began to grow in primary culture, became confluence or contaminated for every procedure and seeding and cell yield of every subculture were noted and tabulated. The procedure that yielded 40-80% confluence in the shortest time was regarded as the best.

RESULTS

Adipose tissue weight in the first (followed by the third) and second procedure was 2.74 and 1.94 grams respectively. The total number of cell yield in procedure 1, 2, and 3 were 60,000, 144,000, and 296,000 cells respectively, and cell yield per gram of adipose tissue in procedure 1, 2 and 3 was 21,898, 74,227, and 108,029 cells, respectively.

The cells were attached at day-3 to day-8 (Table 1). Primary culture took a long time to grow, but subcultures grew faster and became confluence in shorter time. The time needed until confluence or contamination, and the total yield of cells for every culturing and sub-culturing procedure can be seen in Table 2.

Cell yield in procedure 1, 2 and 3 was 21,898, 74,227, and 108,029 cells/gram of adipose tissue.

Some of the cultures were contaminated, so that further subculture was not applicable, and only one tissue processing procedure could complete the three subcultures (Table 1 and 2).

Table 1. The number of seeded cells and needed time for attachment

Procedure	Seeding (final)	Cell attachment	Cell growth	Cell yield
1-1	24,000	Day-8	NC-42	-
1-2	36,000	Day-8	NC-18	-
2-1	24,000	Day-7	NC-42	-
2-2	48,000	Day-3	C-16 (70%)	200,000
2-3	72,000	Day-3	C-16 (40%)	32,000
3-1	74,000	Day-5	C-28 (80%)	1,464,000
3-2	74,000	Day-6	NC-13	-
3-3	148,000	Day-7	NC-11	-

C-= confluence at day-, NC= not yet confluence and contaminated at day-

Table 2. The time needed until sub-culturing or contamination and the yield of cells for every sub-culturing in the various procedures

P	Subc-1	Cell yield (x1000)	Subc-2	Cell yield (x1000)	Subc-3	Cell yield (x1000)
1-1	NA	-	NA	-	NA	-
1-2	NA	-	NA	-	NA	-
2-1	NA	-	-	-	-	-
2-2	Dir-S: 100, C-7 (80%)	1,080	Dir-S: 540, C-8 (90%)	4,496	Dir-S: 1,124, C-7 (80%)	2,960
			W-S: 152, C-15 (50%)	460	NA-cryo	-
	W-S: 60, C-7 (50%)	456	Dir-S: 228, C-8 (80%)	1,152	Dir-S: 384, C-7 (80%)	2,464
			W-S: 80, Cont-21 (40%)	144	NA	-
2-3	Dir-S: 16, C-23 (85%)	1,312	NA - cryo	-	NA	-
	W-S: 8, Cont-22	-	NA	-	NA	-
3-1	Dir-S: 488x1.5, cont-1	-	NA	-	NA	-
3-2	NA	-	NA	-	NA	-
3-3	NA	-	NA	-	NA	-

P= procedure, Subc= subculture, C-= confluence at day-, cont-= contaminated at day-, NA= not applicable, Dir-S= directly sub-cultured and number of cell seeded, W-S= washed before sub-cultured and number of cell seeded, cryo= cryopreserved

DISCUSSION

In this preliminary study, we studied various simple procedures and modified them to fit with the equipments in our lab. Two out of the three modified procedures tested in this study yield confluence culture and could be harvested. Though not all of the procedures yielded harvested cells, conclusion for primary culture still can be drawn. In primary culture, initial seeding of far less than 50.000 cells needed longer time to become confluence as can be seen in procedure 1.1, 1.2, and 2.1 (Table 1). The longer the time needed, the more medium change should be done, and thus the higher risk of contamination. Some studies seeded 1,000,000-5,000,000 nucleated cells/cm2 for primary culture to reach confluence state faster. [8,9]

Theoretically, the more the amount of seeded cells, the shorter is the time that is needed for the culture to become confluence. However, procedure-3 with 74,000 seeded cells needed a longer time (80% confluence at day-28, whereas at day-16 was far from confluence) compared to procedure-2 with 48,000 and 72,000 seeded cells, which both become 70% and 40% confluence at day-16, though the first yield more harvested cells (Table 1). Procedure-3 with 144,000 seeded cells might give better result if it was not early contaminated. The longer time to confluence that was needed by procedure-3 compared to procedure-2 might be due the difference in cell viability, as the overall digestion of adipose tissue on procedure-3 took a longer time (30+45 minutes), and after digestion, the cells were subjected to treatment with lysis buffer. Treatment with lysis buffer after 45 minute digestion was suggested by a study, to eliminate the red blood cells that might interfere with the growth of stem cells.[8] However, this preliminary study showed that lysis buffer treatment after 75 minute digestion was deleterious to the cells, as was shown by the longer time needed to become confluence. The number of seeded cells might be more, but the number of viable cells might be less than in procedure-2. Therefore, if lysis buffer treatment will be used, digestion should not exceed 45 minutes.

Procedure-1 yielded the less of cells, as the time of digestion was the shortest. Moreover, the cells from stromal-vascular fraction might be fewer than other procedures, as shorter digestion might not reach enough of the stromal compartment, so that most of the cells might not be the stem cells that readily grow in culture. Adipose tissue derived stem cells that grow well in culture are supposed to be pericytes that are found in the stromal-vascular fraction.[8-10] Looking at the result of procedure-1 with 24,000 seeded cells that did not become confluence at day-42 lead to a conclusion that in our lab condition, 30 minute digestion was not enough to get the desirable cells in sufficient amount to grow well in primary culture. However, a study showed that 30 minutes digestion was enough.[2] Another study on lipoaspirate sample used an orbital shaker and 30 minutes digestion with good result.[11] Using a shaker provides continuous mixing compared to gently shaking every 5 minutes as was done in our lab, and thus better digestion.

In our study, subculture procedure without washing always gave more cells at harvest (Table 2), as washing always caused loss of cells. Therefore, in subculture-3, all subculture procedures were done without washing step. The washing step was intended to minimize the deleterious effect of trypsin. However, in our experience washing step caused too much cell loss, and the overall result showed that the procedure without washing step gave better result. Moreover, the trypsin that was used to detach the cells was neutralize by the serum in the TC medium, and the next day the TC medium was replaced by fresh TC medium. Subculture without washing step was used in many studies.[8-10]

In conclusion, in this preliminary study, 60 minute colagenase-1 digestion with intermittent shaking every 5 minutes and cell seeding around 50.000 or more, followed by subculture method without washing step gave the best result

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