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EFFECT OF DIFFERENT CULTURE MEDIUM ON CULTIVATION OF ADIPOSE TISSUE DERIVED STEM CELLS FROM TWO BIOLOGICAL SOURCES

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ABSTRACT

Aim of this study was to optimize the methodology of isolation and cultivation of rabbit adipose derived stem cells. Visceral fat and subcutaneous fat from interscapular region of the New Zealand White Line rabbit were used for experiments. Stem cells were cultured in αMEM and DMEM medium supplemented with 20% FBS and 1% antibiotics. We created 4 experimental groups based on source of fat tissue and cultivation medium: Interscapular_αMEM, Interscapular_DMEM, Visceral_αMEM, Visceral_DMEM. Using flow cytometry we analyzed viability of stem cells and intracellular and surface markers: vimentin, desmin, αSMA, CD29, CD34, CD44 and CD45. Morphology analyses revealed typical fibroblastic-like spindle shape cells in all cultured groups after few days of culture. Results of our study confirmed positive expression of CD29 and CD44 in all experimental groups thereby we confirmed that adipose derived stem cells have mesenchymal origin. Cells also showed positive expression of intracellular markers. Typical hematopoietic markers CD34 and CD45 were not expressed. Statistical analysis did not show significant differences between individual experimental groups, but based on tendency to fasten cell growth (recorded by regular observation and population doubling time analysis), we selected Interscapular_αMEM as a group for further experiments.

Keywords: rabbit, adipose-derived stem cells, flow cytometry

INTRODUCTION

Adipose tissue, as a source of stem cells, has several advantages over bone marrow such as relatively easy sampling or a significantly higher incidence of stem cells in fat tissue compared to bone marrow (2% against 0.002%) **(Strem and Hedrick, 2005)**. Adipose tissue can be easily obtained percutaneously from subcutaneous tissue or by aspiration techniques **(Park *et al.*, 2010)**. The amount of pluripotent cells per milliliter of adipose tissue is variable, depending on the animal species. For instance, the amount of adipose derived stem cells (ADSCs) in one milliliter of adipose tissue in rabbits is lower than in rats or pigs **(Arrigoni *et al.*, 2009).**

Fat tissue is becoming an increasingly important source of stem cells, mainly due to the growing obesity in the world, which greatly facilitates the acquisition of this tissue **(Gimble *et al.*, 2007).** The great advantage of these stem cells is their multitude and availability compared to other sources of mesenchymal stem cells (MSCs) **(Zuk *et al.*, 2001).** ADSCs are morphologically similar to mesenchymal stem cells derived from bone marrow or other tissues. These cells have the ability to differentiate into cells of mesenchymal origin such as adipocytes, myocytes, chondrocytes and osteocytes **(Cheng *et al.*, 2011)**.

ADSCs are a new and effective tool to promote bone regeneration. **De Girolamo *et al.* (2011)** used twelve New Zealand White rabbits to harvest fat tissue and isolate stem cells from it in their study. The results demonstrate that these cells show a high differentiation potential and can be used to treat bone defects. **Bunnel *et al.* (2008)** reported that human adipose tissue is an abundant and easily accessible source of adult stem cells with multipotent properties that are suitable for tissue engineering and regenerative medicine.

Obviously, there are key similarities and differences between ADSCs and stem cells isolated from bone marrow. For example, CD105, STRO-1 and CD166 are three common markers that are used to identify cells with a multilinear differentiation potential and are expressed both in bone marrow derived stem cells and in ADSCs **(Strem and Hedrick, 2005)**. Based on current MSCs research, stem cells have to meet three criteria to qualify as mesenchymal. The first criterion is adherence to plastic under standard culture conditions; the second criterion is that cells must be capable of differentiation into osteocytes, chondrocytes and adipocytes. The last condition is the expression of surface markers CD29, CD44, CD90 and, at the same time, mesenchymal cells should not express markers CD34 and CD45 **(Alipour *et al.*, 2015)**.

The aim of present work was to compare the harvestability of stem cells from two sources of adipose tissue (interscapular and visceral) and their further cultivation in two different media (αMEM and DMEM).

MATERIAL AND METHODS

Biological material

The rabbits (n=8) of New Zealand White Line reared in a partially air-conditioned hall of rabbit farm of the Institute of Small Farm Animals of the Research Institute of Animal Production Nitra in Lužianky were used for the experiment. The rabbits were housed in individual cages, at a constant photoperiod of 14 hours of light day. Temperature and humidity were recorded using a thermograph placed in the height of the cages (the average humidity and temperature during the year is maintained at 60 ± 5% and 17 ± 3 ° C). The rabbits were fed *ad libitum* with a commercial feed mixture (KV, TEKRO Nitra, s.r.o.) and the water was provided *ad libitum* using water feeders. Animal treatment was approved by the State Veterinary and Food Administration of the Slovak Republic no. SK CH 17016 and SK U 18016.

Harvesting and processing of the adipose tissue

Rabbits weighing approximately 3 - 4.5 kg were humanely sacrificed and subsequently visceral and subcutaneous fat was harvested. The collected fat samples were washed with PBS containing 5% antibiotics penicilin and streptomycin (Life Technologies, Slovak
Republic). Following washing, debris (blood vessels, connective tissue, muscle tissue, etc.) was removed using the scissors and tweezers. Adipose tissue was cut into small pieces and was rewashed with PBS containing antibiotics. Samples were centrifuged for 5 minutes at 500 x g.

Isolation and cultivation of stem cells from adipose tissue

Tissue samples were incubated at 37 ° C for about 2 hours with collagenase type I (Sigma Aldrich, UK) at a concentration of 0.075% for visceral fat and 0.2% for subcutaneous fat. The tissue solution was neutralized with culture medium and

filtered through a 100 µm filter to remove the undigested tissue. After filtration, the samples were centrifuged for 10 minutes at 1200 x g. Following centrifugation, cell pellets were resuspended in DMEM (Gibco-BRL, USA) and αMEM (Gibco-BRL, USA) culture medium with 20% FBS and 1% antibiotics. Cells were cultured at 37°C and 5% CO_2. The medium was replaced after 24 hours to remove non-adherent cells and later changed every second day. After reaching 80-90% confluency cells were detached using 0.05% Trypsin-EDTA (Thermo Fisher Scientific, USA) for 5 minutes at 37°C and 5% CO_2. The cell concentration was counted using the EVE™ Automated cell counter (NanoEntek, USA). Cells were seeded at a density of 12 x 10^3 / cm^2. Cells were cultured till the passage 3.

Population doubling time

In order to determine the population doubling time (PDT) cells of all experimental groups were counted at every passage (P1 – P3) and culture time was recorded. Briefly cells were dissociated using 0.05% Trypsin-EDTA for 5 min and following centrifugation (at 200 x g for 5min), cells were resuspended in 1 ml of culture media. Cell concentration was counted using EVE™ Automatic Cell Counter following 0.4% Trypan Blue staining and loading into EVE cell counting slide. Population doubling time (PDT) was calculated for each passage by the growth curve using doubling time calculator available at http://www.doubling-time.com/compute.php.

Flow cytometry analyses

Detection of cell surface markers and intracellular markers were performed using flow cytometry antibody staining as described in previous study of **Kovac *et al.* (2017)**. To analyze CD markers, 5 million cells (P3) were taken from the cell suspension. Prior to surface proteins analysis, pellets were resuspended in 50 µl rabbit serum to block Fc receptors. Cell aliquots were then incubated with primary antibodies: anti-rabbit CD29 in FITC (Merck, Slovak Republic), anti-human CD34 in FITC (Thermo Fisher Scientific, USA), anti-rabbit CD44 (Bio-Rad, UK), anti-rabbit CD45 (Bio-Rad, UK). Double staining by proper fluorochrome conjugated secondary antibodies was performed using rat anti-mouse IgG1-PE (clone X-56; Miltenyi Biotec, Germany).
For the purpose of intracellular staining 4 million cells were taken from cell suspension (P3). Intracellular staining of vimentin, desmin and α-SMA (alpha – smooth muscle actin) was performed as described by **Kovac *et al.* (2017)**.

Analyses of viability

Viability and apoptosis rates were evaluated using specific fluorescent probes as follows: Yo-Pro-1 (Molecular Probes, Switzerland) to detect early apoptosis and propidium iodide (PI) to detect necrotic cells (Molecular Probes, Switzerland), respectively. Cells were incubated with these dyes as in detail portrayed in previous study **(Kovac *et al.*, 2017)**. Cells were then analysed using flow cytometer FACS Calibur™ (BD Biosciences, USA).

Statistical analysis

The results obtained by flow cytometry were processed applying the One Way ANOVA (Tukey assay) using the SigmaPlot software (Systat Software Inc., Germany) and expressed as ± SEM (standard error of the mean)

RESULTS AND DISCUSSION

Isolation and culture of adipose tissue derived stem cells

Based on the source of fat and media four experiment groups were established: Interscapular_αMEM, Interscapular DMEM, Visceral αMEM, Visceral_DMEM. In the first day of culture cells with round shape were observed. After 24 hours of isolation, cells started to adhere to tissue culture flasks and morphology changed into spindle-shaped. Medium was replaced every 2 days in order to remove non-adherent cells. After 6-7 days the cells reached 80-90% confluency and the culture consisted of homogenous monolayer of fibroblast-like cells. Based on the regular observations, we concluded that cells grew faster in group Interscapular_αMEM. Similar tendency was recorded by the analysis of population doubling time. PDT was calculated according to cell numbers counted after detachment and culture time. An average PDT for ADSCs in our study was **29.8±4.1 hours**. No difference was found among the groups (Figure 2).

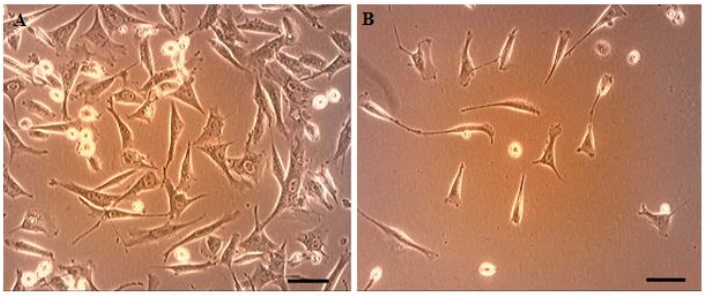

Figure 1 Morphology of adipose derived stem cells four days post isolation. (A) Interscapular_αMEM. (B) Interscapular_DMEM. Scale bar = 200µm.

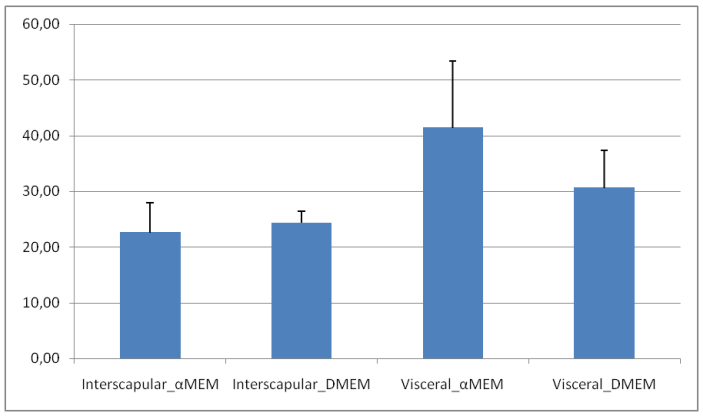

Figure 2 Population doubling time of ADSCs [hours]

ADSCs viability

Stem cell viability was evaluated from the third passage (P3) from internal and subcutaneous fat samples. Samples were evaluated for the percentage of apoptotic cells (YoPro) and percentage of necrotic cells (PI). There was no statistically significant difference among the groups (Figure 3).

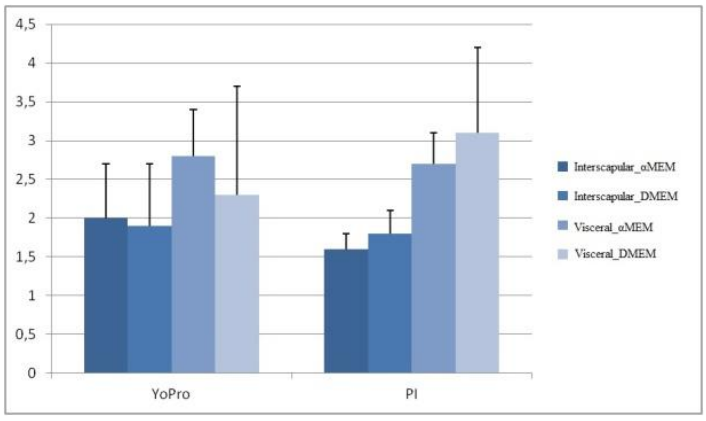

Figure 3 ADSCs viability; YoPro- apoptotic cells, PI- necrotic cells [%]

Flow-cytometric characterization

Using flow cytometry, four markers were tested on samples, two of which are characteristic for ADSCs (CD29 and CD44). Markers CD45 and CD34, characteristic for hematopoietic cells, were tested on samples as a negative control. The results of present work confirmed the high expression of CD29 and CD44 markers in all four groups, regardless of culture medium or fat source. Isolated ADSCs did not express the CD34 and CD45 markers in either experimental group, thus confirming that the isolated stem cells are of mesenchymal origin. There was no statistically significant difference among the experimental groups. Detailed results of surface markers expression are shown in Table 1.

Table 1 ADSCs surface markers expression

	Interscapular αMEM	Interscapular DMEM	Visceral αMEM	Visceral DMEM
CD29 (%)	88.9±6.9	96.3±1.9	78.4±7.5	84.1±6.9
CD44 (%)	95.3±1.6	88.8±6.0	90.9±2.4	96.9±1.8
CD34 (%)	0.3±0.1	0.5±0.1	0.5±0.1	0.6±0.01
CD45 (%)	1.6±0.8	1.6±0.3	2.6±0.6	1.8±0.3

Results are expressed as mean ± SEM.

In order to analyze the rabbit ADSCs phenotype, intracellular markers Vimentin, Desmin, α-SMA were assessed by flow cytometry analysis. Statistical analysis of the results did not show a significant difference between the tested culture media (αMEM, DMEM), or between fat sources (interscapular fat, visceral fat). The measured values of intracellular markers expression are shown in Table 2.

Table 2 ADSCs intracellular markers expression

	Interscapular αMEM	Interscapular DMEM	Visceral αMEM	Visceral DMEM
Vimentin (%)	88.7±7.1	90.8±3.4	78.4±6.7	96.2±1.0
Desmin (%)	83.7±7.9	79.8±5.1	73.1±8.9	90.7±4.3
α-SMA (%)	91.6±4.5	91.4±3.3	82.7±6.1	95.7±2.6

Results are expressed as mean ± SEM.

Previous analyzes of ADSCs surface markers expression demonstrate that these cells express similar surface markers as stem cells isolated from bone marrow **(Gronthos *et al.*, 2001; Katz *et al.*, 2005)**. **Li *et al.* (2017)** reported similar results in rabbit stem cells isolated from the subcutaneous tissue. Using flow cytometry, the surface markers expression was moderately higher than in present work (CD29 - 97.3 ± 1.2% and CD44 - 98.1 ± 0.7%). Conversely, in the negative controls for CD34 and CD45 markers, the CD34 values were 4.8 ± 0.6 and CD45 - 4.3 ± 0.9, which is higher positivity than results found in our study. In mentioned work, was also highlighted that markers CD54, CD73, CD90 and CD105 had high positivity (>90%).

Tsekouras *et al.* (2017) assessed the viability and expression of ADSCs markers on cells isolated by liposuction from different body areas of women. The results of their work point out that the area from which the fat is harvested is a very important factor for the quality of stem cells in terms of viability. The authors compared fat from five areas: abdomen, waist, inner thigh, outer thigh and knee. From the point of view of the viability and yield of the cells, the thighs were confirmed as the best source. When assessing the cell phenotype, no significant differences between groups were indicated.

Media conventionally used for isolation and culture of ADSCs are usually supplemented with 10% FBS **(Suga *et al.*, 2007)**. Differences among doses that may affect the stem cell phenotype are well known **(Caterson *et al.*, 2002)**. **Lee *et al.* (2014)** compared the expression of the surface and intracellular markers of stem cells isolated from bone marrow and adipose tissue in humans and rabbits. Samples of subcutaneous adipose tissue were cultured in DMEM medium with the addition of 10% FBS to the third passage. The results of their work point out that the phenotype of ADSCs is similar, although not identical to the phenotype of stem cells from bone marrow. The difference between rabbit and human stem cells was obvious. Compared to present results, no or lower expression of markers CD29 (1.47 ± 0.86) and desmin (31.32 ± 4.83) was detected in their study. Expression of surface markers in horse adipose tissue derived stem cells was investigated by **Alipour *et al.* (2015)**. Expression was monitored using PCR which confirmed the expression of markers CD29, CD44, CD90.

Riis *et al.* (2016), in their work, also dealt with the comparison of the effects of different media on cell proliferation, morphology and phenotype of human ADSCs. Three different media were tested in the study: DMEM, αMEM and StemPro. The results suggested that the best cell grow was observed in αMEM medium. The StemPro medium was proved to be unsuitable mainly due to low cell growth initiation, DMEM promoted the cell proliferation, however in smaller extent than αMEM. Results of this study is in line with our results because we also found that αMEM medium is more suitable according to morphological observation of cell growth. Based on these results, the analysis of surface markers was applied only on cells cultured in αMEM only. Selected surface markers (CD73, CD90 and CD105) showed the level of expression over 80%.

The fact that the phenotype of ADSCs is similar to the phenotype of MSCs from adipose tissue was confirmed in study of **Musina *et al.* (2005)**, who in addition to fat tissue, also compared stem cells isolated from bone marrow, skin and placenta. For all sources, the high expression of the CD44 marker, characteristic for these cells, has been confirmed. This marker was expressed in 98.9% of the monitored cells from adipose tissue.

CONCLUSION

The aim of present work was to optimize the methodology of isolation and cultivation of rabbit stem cells from adipose tissue. The study was focused on comparison of two sources of fat tissue and two culture media. The results of the work suggest the αMEM medium as more suitable for rabbit ADSCs according to cell growth observation results. The cells were spindle-shaped, what varied in dependence with cell confluency. Analyses of viability and marker expression did not show a statistically significant difference between the individual experimental groups. In all groups, the expression of the surface markers CD29 and CD44 was confirmed, proving that ADSCs have mesenchymal origin. Isolated stem cells also expressed intracellular markers vimentin, desmin and α-SMA. In conclusion, rabbit adipose tissue showed to be a promising source of stem cells that could be preserved as a genetic resource in animal gene bank. Based on obtained results the αMEM medium was chosen for future analyses on adipose derived stem cells.

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