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RABBIT ADIPOSE-DERIVED STEM CELLS MAINTAIN THEIR CHROMOSOMAL COUNTS DURING PASSAGING: SHORT COMMUNICATION

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ABSTRACT

Monitoring of stem cells genetic stability is one of the most important safety points, because the number of chromosomes can change throughout the culture and *in vitro* manipulation of these cells. In our study the stem cells metaphases were analysed using the G-staining method. At least 60 metaphases in three samples of rabbit adipose-derived stem cells were assessed in three subsequent passages. Results of our study showed that at least 70 % of cells in each passage can maintain their stable karyotype. The highest proportion of aneuploidy (30 %) was recorded in the third passage. Even though we observed a slight increase of aneuploidy during passaging, statistical analysis did not show any significant differences. Based on our results, we can conclude that cell passaging does not affect genetic stability, since there were no changes in chromosomal counts throughout the culture. However, it was observed that there may be some instabilities during passaging that are more random. For this reason, it is recommended to monitor the stem cells' karyotype, especially if they are intended for therapeutic use.

Key words: rabbit; adipose derived stem cells; chromosomes; karyotype

INTRODUCTION

Latterly, adipose tissue has been a considerable source of mesenchymal stem cells due to its easy harvesting, accessibility and abundance of cells (Du *et al.*, 2010; Kim *et al.*, 2011). Stem cells from adipose tissue (ASCs) have many similarities, such as morphology, expression of surface markers and differentiation potential with stem cells obtained from other tissues of bone-marrow or umbilical cord origin. Mesenchymal stem cells (MSCs) from adipose tissue are nowadays considered as a widespread material for a variety of clinical applications, thus their use entails continuous safety monitoring (Kern *et al.*, 2006; Neri *et al.*, 2013).

One of the important safety points is the genetic stability that may change during culture and *in vitro* manipulation of cells. MSCs are often cultured to obtain sufficient amount for cells therapies, which increases the presumption that genetic changes may arise (Casiraghi *et al.*, 2013). There are several studies which proved that *in vitro* cell production can lower replicative potential and multipotency and that in fact leads to senescence of cells; it reduces DNA polymerase and DNA repair efficiencies, thus leading to enhanced DNA damage, such as cytogenetic alterations (deletions, duplications), mutations and epigenetic changes (Bonab *et al.*, 2006; Sperka *et al.*, 2012; Neri *et al.*, 2013). Adipose-derived stem cells in culture may be subject to chromosomal mutations and therefore changes in karyotype can be observed. It has not been specified yet in what culture conditions the mutations are the most frequent and the results

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obtained by independent research groups are often contradictory (Ferreira *et al.*, 2012).

Analyse of karyotype is very useful tool to monitor the genetic stability of ASCs during culture. Currently there is a lot of different techniques for the purpose of karyotype investigation. Each method has advantages and disadvantages in terms of sensitivity and financial point of view (Catalina *et al.*, 2007, Čurlej *et al.*, 2018). Therefore, even not very expensive method may contribute to achievement of very valuable information about genetic aspect of examined cells.

The aim of our study was to analyse the karyotype of rabbit adipose-derived stem cells in order to assess the effect of culture conditions on genetic stability of ASCs.

MATERIAL AND METHODS

Biological material

Clinically healthy rabbits (n = 3) of New Zealand White line were used in the study. Rabbits were reared in a partially air-conditioned rabbit farm hall of the Institute of Small Farm Animals at the NPPC – Research Institute for Animal Production Nitra, Slovakia. Rabbits were housed in individual cages, at a constant photoperiod of 14 hours of light a day. Temperature and humidity were recorded using a thermograph placed at 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* a commercial feed mixture (KV, TEKRO Nitra, Slovakia) 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, weighting approximately 3 – 4.5 kg, were humanely sacrificed and subsequently subcutaneous fat was harvested. The collected fat samples were washed with PBS (without Ca and Mg; Biowest, USA) containing 5 % antibiotics penicillin and streptomycin (Life Technologies, Slovak Republic). Following washing, debris (blood vessels, connective tissue, muscle tissue, etc.) was removed using 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 adipose derived stem cells

Tissue samples were incubated at 37 °C for about 2 hours with collagenase type I (Sigma Aldrich, UK) at concentration of 0.2 %. 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 αMEM (Gibco-BRL, USA) culture medium supplemented with 20 % fetal bovine serum (Sigma Aldrich, UK) and 1 % antibiotics. Medium was changed every 3 days in order to remove non-adherent cells. Cells reached 90 % confluency in about 6-7 days after isolation. We seeded cells at a density of 12 x 10^3 / cm^2. Cells were cultured until the passage 3 (P3), as previously described in our study (Tomková *et al.*, 2018).

Karyotype analysis

Samples for chromosome analysis were processed as follows. After passaging, actively growing cells from P3 were incubated with KaryoMAX® Colcemid™ solution in HBSS (Hanks' Balanced Salt Solution, Life Technologies, Slovak Republic) for 16 h at 37 °C and 5 % CO_2. Treated cells were washed with Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, USA) and dissociated by 0.05 % trypsin (ThermoFisher, Slovak Republic). After centrifugation (200 x g for 10 min), cells were resuspended and incubated in a hypotonic solution (75 mM KCl) for 20 min at 37 °C. Following centrifugation, the harvested cells were incubated with 5 ml of chilled fixative consisting of methanol and acetic acid (3:1; v/v) for 10 minutes. Chromosome spreads were prepared by dropping the cell suspension onto pre-chilled glass slides. The air-dried cell spreads were stained by 2 % Giemsa solution (Gibco BRL, USA) and observed under a light microscope at 400 x magnification.

Statistical analysis

A normal rabbit karyotype consists of 22 pairs (2n = 44) of chromosomes. Chromosomal abnormality was defined as following: hyperploidy -> 44, hypoploidy - < 44. The final percentage of abnormal karyotype was defined as (< 2n) + (> 2n). Statistical

analysis was performed with χ^2–test for comparison of percentages using Excel software.

RESULTS AND DISCUSSION

Following 24 hours after isolation cells started to adhere to culture flasks and they showed a typical spindle-shaped morphology (Figure 1). As the cells reached the passage 3, the karyotype analysis was performed.

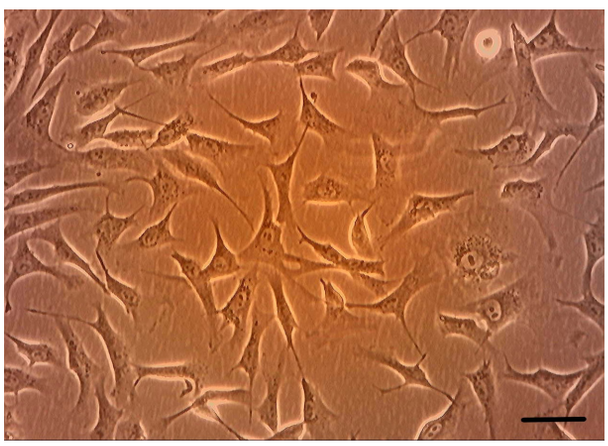

Figure 1. Spindle-shaped morphology of adipose-derived stem cells. Scale bar = 200µm

Results of karyotype analyses showed that more than 70 % of cells in all samples can maintain their chromosomal counts in each passage (Figure 2). The highest proportion of aneuploidy (30 %) was recorded in sample 2 in the third passage. In our study, we did not observe significant changes in chromosomal counts during passaging, however there were some abnormalities observed in each passage (Tables 1 – 3). The results of our study are comparable to Bellotti *et al.* (2013), who observed karyotype of adipose-derived stem cells at passages 2, 7 and 15. Abnormalities in karyotype were observed mainly at passage 15, suggesting that longer-term cultivation may result in changes in the genetic stability of cells due to cell aging.

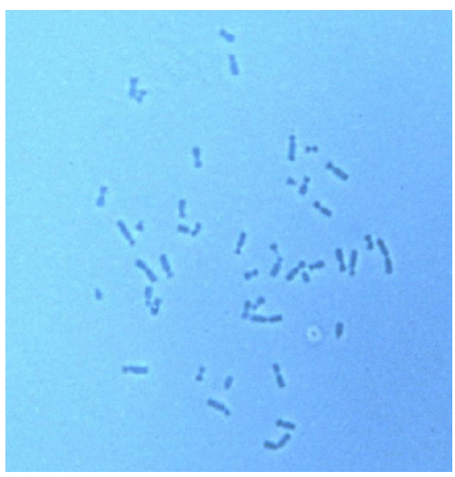

Figure 2. Normal karyotype of rabbit adipose-derived stem cells

Table 1. Chromosomal counts in passage 1

P1	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Sample 1	60	81.67 / 49	11.67 / 7	6.66 / 4	18.33
Sample 2	60	76.67 / 46	15.00 / 9	8.33 / 5	23.33
Sample 3	60	83.33 / 50	13.33 / 8	3.34 / 2	16.67

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoploidy; > 2n = aneuploidy: hyperploidy

Table 2. Chromosomal counts in passage 2

P2	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Sample 1	60	80.00 / 48	11.67 / 7	8.33 / 5	20.00
Sample 2	60	73.33 / 44	11.67 / 7	15.00 / 9	26.67
Sample 3	60	81.67 / 49	10.00 / 6	8.33 / 5	18.33

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoploidy; > 2n = aneuploidy: hyperploidy

Table 3. Chromosomal counts in passage 3

P3	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Sample 1	60	76.67 / 46	13.33 / 8	10.00 / 6	23.33
Sample 2	60	70.00 / 42	16.67 / 10	13.33 / 8	30.00
Sample 3	60	78.33 / 47	10.00 / 6	11.67 / 7	21.67

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoploidy; > 2n = aneuploidy: hyperploidy

Based on our results we can conclude that cells passaging does not affect genetic stability, since there are no changes in chromosomal counts in culture. However, it has been already observed that during passaging there may be some instabilities that are more random. This is confirmed by study of Binato *et al.* (2012), who examined karyotype of human stem cells isolated from bone marrow. In his study, the cells maintained a stable karyotype for up to 4 passages. In the higher passages, Binato *et al.* (2012) also reported an accidental occurrence of aneuploidy.

Majority of current studies claims MSCs have stable karyotypes (Meza-Zepeda *et al.*, 2008; Ben-David *et al.*, 2011; Sensebé *et al.*, 2012; Stultz *et al.*, 2016). Similar results were also obtained in our previous studies on stem cells isolated from different tissues as bone marrow (Vašíček *et al.*, 2020), endothelial progenitor cells (Tomková *et al.*, 2017) or amniotic fluid (Kováč *et al.*, 2017; Kulíková *et al.*, 2019).

CONCLUSION

In our study we confirmed the relevance of cytogenetic studies, especially for the purpose of monitoring of numeric chromosomal aberrations. Abnormalities in chromosomal counts were detected in all samples throughout culture but no significant differences were proven so we concluded that these instabilities are more random.

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REFERENCES

Bellotti, C., Stanco, D., Ragazzini, S., Romagnoli, L., Martella, E., Lazzati, S., Marchetti, C., Donati, D. & Lucarelli, E. (2013). Analysis of the karyotype of expanded human adipose-derived stem cells for bone reconstruction of the maxillo-facial region. *International Journal of Immunopathology and Pharmacology*, 26(1 Suppl), 3–9.

Ben-David, U., Mayshar, Y., & Benvenisty, N. (2011). Large-scale analysis reveals acquisition of lineage-specific chromosomal aberrations in human adult stem cells. *Cell Stem Cell*, 9(2), 97–102.

Binato, R., de Souza Fernandez, T., Lazzarotto-Silva, C., Du Rocher, B., Mencalha, A., Pizzatti, L., Bouzas, L. F. & Abdelhay, E. (2013). Stability of human mesenchymal stem cells during *in vitro* culture: considerations for cell therapy. *Cell Proliferation*, 46(1), 10–22.

Bonab, M. M., Alimoghaddam, K., Talebian, F., Ghaffari, S. H., Ghavamzadeh, A., & Nikbin, B. (2006). Aging of mesenchymal stem cell *in vitro*. *BMC Cell Biology*, 7(1), 14.

Catalina, P., Cobo, F., Cortés, J. L., Nieto, A. I., Cabrera, C., Montes, R., Concha, A. & Menendez, P. (2007). Conventional and molecular cytogenetic diagnostic methods in stem cell research: a concise review. *Cell Biology International*, 31(9), 861–869.

Casiraghi, F., Remuzzi, G., Abbate, M., & Perico, N. (2013). Multipotent mesenchymal stromal cell therapy and risk of malignancies. *Stem Cell Reviews and Reports*, 9(1), 65–79.

Curlej, J., Tomková, M., Vasicek, J., & Chrenek, P. (2018). Cytogenetic studies of mesenchymal stem cells in rabbit: A mini-review. *Slovak Journal of Animal Science*, 51(4), 150–155.

Du, Y., Roh, D. S., Funderburgh, M. L., Mann, M. M., Marra, K. G., Rubin, J. P., Li, X. & Funderburgh, J. L. (2010). Adipose-derived stem cells differentiate to keratocytes *in vitro*. Molecular Vision, 10(16), 2680–2689.

Ferreira, R. J., Irioda, A. C., Cunha, R. C., Francisco, J. C., Guarita-Souza, L. C., Srikanth, G. V., Nityanand, S., Rosati, R., Chachques, J. C. & de Carvalho, K. A. (2012). Controversies about the chromosomal stability of cultivated mesenchymal stem cells: their clinical use is it safe? *Current Stem Cell Research & Therapy*, 7(5), 356–363.

Kern, S., Eichler, H., Stoeve, J., Klüter, H., & Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells*, 24(5), 1294–1301.

Kim, J. H., Jung, M., Kim, H. S., Kim, Y. M., & Choi, E. H. (2011). Adipose-derived stem cells as a new therapeutic modality for ageing skin. *Experimental Dermatology*, 20(5), 383–387.

Kovac, M., Vasicek, J., Kulikova, B., Bauer, M., Curlej, J., Balazi, A., & Chrenek, P. (2017). Different RNA and protein expression of surface markers in rabbit amniotic fluid-derived mesenchymal stem cells. *Biotechnology Progress*, 33(6), 1601–1613.

Kulikova, B., Kovac, M., Bauer, M., Tomkova, M., Olexikova, L., Vasicek, J., Balazi, A., Makarevich, A. V. & Chrenek, P. (2019). Survivability of rabbit amniotic fluid-derived mesenchymal stem cells post slow-freezing or vitrification. *Acta Histochemica*, 121(4), 491–499.

Meza-Zepeda, L. A., Noer, A., Dahl, J. A., Micci, F., Myklebost, O., & Collas, P. (2008). High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. *Journal of Cellular and Molecular Medicine*, 12(2), 553–563.

Neri, S., Bourin, P., Peyrafitte, J. A., Cattini, L., Facchini, A., & Mariani, E. (2013). Human adipose stromal cells (ASC) for the regeneration of injured cartilage display genetic stability after *in vitro* culture expansion. *PloS One*, 8(10), e77895.

Sensebé, L., Tarte, K., Galipeau, J., Krampera, M., Martin, I., Phinney, D. G., & Shi, Y. (2012). Limited acquisition of chromosomal aberrations in human adult mesenchymal stromal cells. *Cell Stem Cell*, 10(1), 9–10.

Sperka, T., Wang, J., & Rudolph, K. L. (2012). DNA damage checkpoints in stem cells, ageing and cancer. *Nature reviews Molecular Cell Biology*, 13(9), 579.

Stultz, B. G., McGinnis, K., Thompson, E. E., Surdo, J. L. L., Bauer, S. R., & Hursh, D. A. (2016). Chromosomal stability of mesenchymal stromal cells during *in vitro* culture. *Cytotherapy*, 18(3), 336–343.

Tomková, M., Vašíček, J., Kulíková, B., Baláži, A., & Chrenek, P. (2017). Comparison of rabbit endothelial progenitor cells and mesenchymal stem cells: cytogenetic approach. *Slovak Journal of Animal Science*, 50(2), 73–76.

Tomková, M., Kulíková, B., Vašíček, J., Baláži, A., Makarevič, A., & Chrenek, P. (2018). Effect of different culture medium on cultivation of adipose tissue derived stem cells from two biological sources. *The Journal of Microbiology, Biotechnology and Food Sciences*, 8(2), 798.

Vašíček, J., Kováč, M., Baláži, A., Kulíková, B., Tomková, M., Olexiková, L., Čurlej, J., Bauer, M., Schnabl, S., Hilgarth, M., Hubmann, R., Shehata, M., Makarevich, A. V. & Chrenek, P. (2020). Combined approach for characterization and quality assessment of rabbit bone marrow-derived mesenchymal stem cells intended for gene banking. *New Biotechnology*, 54, 1–12. (doi: 10.1016/j.nbt.2019.08.001. Epub 2019 Aug 7).