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Original article

Immunophenotypic characteristics and karyotype analysis of bone marrow-derived mesenchymal stem cells of rabbits during in vitro cultivation

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Abstract

The aim of this study was to establish the immunophenotypic profile and karyotypic stability of bone marrow mesenchymal stem cells (MSCs) of rabbits at the early passages in vitro following the application of different methods of dissociation of cellular material. MSCs were obtained from the femur bone marrow of three clinically healthy rabbits under general anaesthesia. Bone marrow aspirate was seeded in Petri dishes and cultured in a CO_2 incubator with 5% CO_2 at 37.0ºC using a standard procedure. Immunohistochemical detection of nuclear proteins, cytoskeletal proteins and cell adhesion were performed by immunohistochemical analysis and karyotype analysis of MSCs following the enzyme and chelating methods of dissociation of the cell monolayer. The results of the immunophenotypic analysis of rabbit bone marrow MSCs showed that at the first, seventh, twelfth, and eighteenth passages these cells express markers of mesenchymal, muscle, epithelial and nerve cells. The choice of the enzyme or chelating method of dissociation of a culture of rabbit mesenchymal stem cells affects their cytogenetic variability. Dissociation of the MSCs monolayer with ethylenediaminetetraacetic acid produces a cell culture with fewer quantitative and qualitative changes in the chromosome apparatus as compared to the enzyme method. Rabbit MSCs express markers of mesenchymal (vimentin, actin), muscle, epithelial and nerve (E-cadherin, N-cadherin) cells that are essential for differentiation of these cells. The chelating method of dissociation of a culture of rabbit mesenchymal stem cells, using ethylenediaminetetraacetic acid during cultivation, is more advantageous than the enzyme method of dissociation because it leads to less cytogenetic variability.

Key words: mesenchymal stem cells, monoclonal antibodies, E-cadherin, N-cadherin, actin, vimentin

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Introduction

Despite great interest in bone marrow somatic stem cells, arising from their multipotency, plasticity, and simplicity of acquisition and use, there are no conventional methods for isolating them from the tissue. Different laboratories use different methods of isolation and purification of these cells, depending on factors such as their size, density, ability to adhere to substrates, or expression of certain surface antigens, and in consequence the cell populations obtained may significantly differ in their characteristics (Chomik et al. 2016).

Valuable information has been obtained during system analysis of cell surface molecules of MSCs (Majumdar et al. 2003). MSCs were found to express a wide range of cell adhesion molecules that are important in cell interaction and migration to sites of tissue damage ('homing'). MSCs exhibit high expression of integrins such as α1, α5, and β1 (CD29); low expression of α2, α3, α6, αV, β2, and β4; and no expression of α4, αL or β2. These data indicate some of the key mechanisms of MSC interaction with other cell types, which, unfortunately, have not yet been investigated in vivo.

Experiments conducted by Pittenger at the end of the 20th century found that multipotent progenitor cells do not have a specific immunophenotype characteristic only for themselves. They express a set of markers characteristic for mesenchymal, endothelial, and muscle cells in the absence of expression of antigens of hematopoietic cells – CD45, CD34 and CD14 (Pittenger et al. 1999).

To facilitate comparison of data obtained by different researchers, in 2006 the MSC Committee of the International Society for Cellular Therapy proposed a set of minimum criteria for assigning cells to multipotent mesenchymal stromal cells (Dominici et al. 2006). However, according to these criteria immunophenotypic characteristics can be applied only to human multipotent mesenchymal stem cells. Thus, investigation of the immunophenotype of animal MSCs is very important for the characterization of cell cultures in vitro. Another extremely important issue in the study of MSCs is cytogenetic analysis of the karyotypic stability of cells during in vitro cultivation to prevent cancer in vivo after their administration. The majority of scientists believe that the emergence of chromosomal abnormalities that characterize the genetic instability of a cell culture are associated with the loss of telomeric DNA and can lead to malignant transformations (Moorhead et al. 1960, Murnane 2012). Some scientific reports indicate that cells with karyotypic changes are not harmful for transplantation because their quantity in the culture is insignificant (Majumdar et al. 2003).

Zhang et al. found that human bone-marrow-derived MSCs do not acquire genetic changes even after 20 days of cultivation (Zhang et al. 2007). Other authors suggest the possibility of spontaneous transformation of MSCs isolated from bone marrow and adipose tissue (Wang et al. 2005), indicating that tumours can arise from these cells in vivo (Serakinci et al. 2004).

The results of cytogenetic studies of umbilical cord MSCs from foals have shown that quantitative changes in chromosomes (aneuploidy and polyploidy) are typical for these cells. In populations of cells from the second to seventh passage an increased number of cells with aneuploidy is observed, indicating rising karyotypic instability of these cells with each successive passage (Mazurkevych et al. 2016).

There may be several reasons, of course, for the conflicting literature data. One is the biological properties of MSCs, and another is the use of different methods of cultivation and passaging of cells. Long-term cultivation of cells using the enzyme method of disaggregation of cellular material can lead to chromosomal aberrations in the MSC culture, while the mechanical method of disaggregation does not cause these anomalies in the karyotype in cell cultures (Buzzard et al. 2004, Inzunza et al. 2004).

Therefore, immunophenotypic research and cytogenetic analysis of mesenchymal stem cells of animals at the early passages during in vitro cultivation has both theoretical and practical importance for veterinary medicine. The purpose of this research was to establish the immunophenotypic profile and karyotypic stability of rabbit bone marrow mesenchymal stem cells at the early passages during in vitro cultivation after application of different methods of dissociation of cellular material.

Materials and Methods

Obtaining rabbit bone marrow and cultivation of mesenchymal stem cells

Mesenchymal stem cells were obtained from the femur bone marrow of three clinically healthy rabbits under general anaesthesia. The animals were sedated by intravenous injection of Xylazinum (3 mg/kg b.w.) and Zoletil was administered intravenously at a dose of 7.5 mg/kg b.w as a general anaesthetic. Bone marrow aspirates were seeded in Petri dishes and cultured in a CO_2 incubator with 5% CO_2 at 37.0°C using a standard procedure. After seeding the MSCs contained in the bone marrow aspirate attached to the bottom of the Petri dishes and proliferated. A suspended culture of hematopoietic stem cells was re-

moved by discarding the culture medium, and cultivation of only those cells that had adhesive properties was continued. The culture media contained 80% Dulbecco' s modified Eagle' s medium and 20% foetal bovine serum with 10 μl/mL antibiotic-antimycotic solution. The culture medium was replaced every 24-48 h. When confluency of the monolayer reached about 80%-90%, the cells were transferred to a suspension using 0.05% trypsin-EDTA solution and reseeded at a ratio of 1 to 3. Microscopic analyses of cell culture quality were carried out using an Axiovert 40 inverted microscope (Carl Zeiss, Germany). The microscopic examination was conducted daily. The material was collected in accordance with the Commission on Bioethics Institute of Veterinary, Medicine NAAS of Ukraine (Resolution N° 1/2013 of 24/01/2013).

Immunophenotypic analysis

Immunohistochemical detection of nuclear and cytoskeletal proteins and cell adhesion was performed by immunohistochemical analysis. The cells were grown on cover glasses for 48-72 h. After the monolayer reached about 50%-70% confluency, the cells were fixed in fixing solution (methanol + acetone, 1:1) for 2 h at 20°C, incubated with a 1% solution of bovine serum albumin (BSA), and treated with monoclonal antibodies-anti-PCNA (clone PC-10, NeoMarkers), Ki-67 (clone RB-9043-PO, Neomarkers), CD44 (clone 156-3C11, DiagnosticBioSystems), PanMuscleActin (clone 1a45C5, DiagnosticBioSystems), E-cadherin (clone SPM 471, ThermoScientific), N-cadherin (clone CD 325, ThermoScientific), vimentin (V9, DiagnosticBioSystems), and CD24 (SN3b, NeoMarkers) for 30-60 min in accordance with the instructions for monoclonal antibody application. The PolyVue visualisation system (ThermoScientific), conjugated with peroxidase, was then used and enzyme activity was detected using diaminobenzidine (ThermoScientific) as a substrate. An immunocytochemical reaction was conducted and the preparations were then washed with water, stained with Mayer haematoxylin (Sigma) for 15-30 s, and placed in Faramount Aqueous Mounting Medium. The results were analysed by counting the number of positively stained cells (brown staining) and evaluated using the classic H-Score method: S = 1xA+ 2xB + 3xC, where S – H-Score index. The values range from 0 (antigen not detected) to 300 (strong expression in 100% of cells); A – percentage of weakly stained cells; B – percentage of moderately stained cells; C – percentage of strongly stained cells.

Cytogenetic analysis

During the karyotypic analysis of the cellular material two series of experiments were performed. In the first series an enzymatic method was used for dissociation of the cell monolayer for passaging of mesenchymal stem cells, adding a 0.25 / 0.02% solution of trypsin/ EDTA to the cells. In the second series of experiments for passaging of mesenchymal stem cells, a chelating method was used for dissociation of the cell monolayer, adding a 0.02% solution of EDTA (ethylenediaminetetraacetic acid) to the cells. Cytogenetic analysis of the cells in both series of experiments was carried out at the first, third and fifth passages. In each cell culture at least 100 metaphase plates were analysed at the first, third, and fifth passage. A modified standard cytogenetic method was used to obtain chromosome preparations (Moorhead et al. 1960).

Fixation of chromosomes was performed 48 h after cell seeding. Colchicine was added to the culture medium at 0.05-0.5 μg/mL and the culture was incubated for 1.5-2 h at 37°C. The cells were removed from the Petri dishes and a cell suspension was obtained by incubation for 1-5 min at 37°C in a solution of trypsin/EDTA or EDTA. The cells were maintained in the metaphase stage by incubation for 30 min at 37°C in a warm hypotonic solution of KCl (0.56%) at a rate of 1 mL of cell suspension per 9 mL of hypotonic solution (1:9). Fixation of chromosomes was performed 3-4 times for 10-20 min in freshly prepared cooled fixative (methanol:glacial acetic acid, 3:1). The chromosome preparations were stained for 40 min with 20% Giemsa stain solution (Merck, Germany). Metaphase plates were analysed using an Axiostar plus microscope (Carl Zeiss, Germany) at 100×and 1,000×.

Quantitative abnormalities of chromosomes, i.e. aneuploidy and polyploidy, as well as structural aberrations, i.e. chromosome and chromatid breaks, were examined. A micronucleus test was also performed, to estimate the number of binucleated cells (BC), cells with micronuclei (CM), and apoptotic cells (AC). The mitotic index (MI) was calculated as well. The frequency of BC, CM, MI, and AC was calculated per 1,000 cells (‰).

Results

Table 1 and Fig. 1a-m present data concerning immunophenotyping of mesenchymal stem cells of rabbit bone marrow at the first, seventh, twelfth, and eighteenth passages. At the first, seventh and twelfth passages the number of PCNA-positive cells was con-

Table 1. Immunophenotypic profile of rabbit bone marrow mesenchymal stem cells at various passages (M ± m, n=3).

Antigen	Passage of cells from rabbit bone marrow in vitro			
	I	VII	XII	XVIII
	Assessment in points using the H-Score method (from 0 to 300)			
Nuclear proteins associated with proliferation and cell cycle				
PCNA	218.3 ± 4.8	201 ± 20	227 ± 14	82 ± 13***
Ki67	108 ± 8.7	180 ± 16**	89 ± 11	18 ± 5***
Proteins of cell adhesion and cytoskeleton				
Vimentin	238.3 ± 18.4	300 ± 0**	264 ± 26	271 ± 24
Actin	163 ± 10	211 ± 22	68 ± 11***	246 ± 19**
E-cadherin	162 ± 12.6	120 ± 10	136 ± 11	132 ± 19
N-cadherin	243 ± 13.5	52 ± 7***	76 ± 4***	141 ± 16**
CD44	247 ± 10	72 ± 13***	89 ± 10***	19 ± 4***

** p<0.01, *** p<0.001

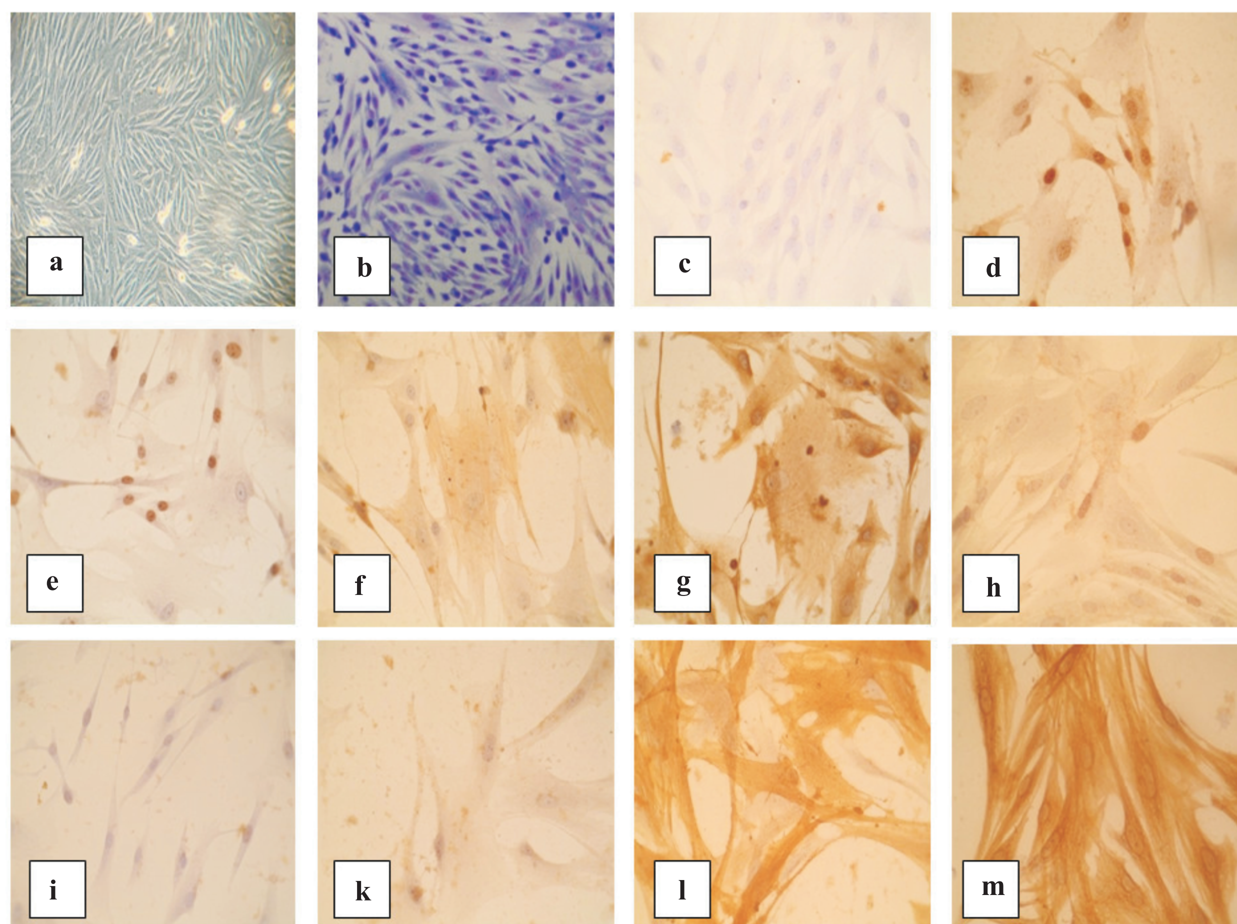

Fig. 1. Culture of rabbit bone marrow mesenchymal stem cells: a – live unstained cell culture (third passage), x100; b – cell culture (third passage) stained using the Pappenheim method, x200; c – control cell culture; d – PCNA-positive cells (seventh passage); e – Ki-67-positive cells (seventh passage); f – actin-positive cells (seventh passage); g – vimentin-positive cells (seventh passage); h – PCNA-positive cells (eighteenth passage); i – Ki-67-positive cells (eighteenth passage); k – E-cadherin-positive cells (eighteenth passage); l – actin-positive cells (eighteenth passage); m – vimentin-positive cells (eighteenth passage), x400.

sistently high, indicating their high proliferative activity, but significantly decreased at the eighteenth passage (Fig. 1d, h). Analysis of antigen Ki-67 expression revealed moderate expression of this protein in the cells at the first passage. It should be noted that at the seventh passage (Fig. 1e) the expression of Ki-67 antigen in the cultured cells was significantly increased in relation to the first passage. However, expression of Ki-67 showed a downward trend at the twelfth passage and was significantly lower at the eighteenth passage (Fig. 1i) as compared to the first passage.

Vimentin is a specific marker of mesenchymal cells and is also a protein of intermediate filaments of the cell cytoskeleton. Examination of vimentin expression in bone marrow cells (Fig. 1g, m) showed a significant increase in the number of vimentin-positive cells (300 points) at the seventh passage. Moreover, high expression of vimentin in cells was noted at the twelfth (264 points) and eighteenth (271 points) passages, with a tendency towards increasing expression in relation to the first passage, which confirms the dominance of cells of mesenchymal origin in the cell culture.

The presence of actin in cells suggests their mesenchymal origin. Analysis of actin expression in cells (Fig. 1f, l) revealed that expression of this protein in cells at the seventh passage had a tendency to increase as compared to cells of the first passage. The immunocytochemical analysis showed that the number of E-cadherin and N-positive cells was significantly decreased in the culture of rabbit bone marrow MSCs at the seventh passage, indicating the inactivation of cell-cell interactions and reduced expression of proteins characteristic of epithelial, neural and skeletal cells. However, the number of E-cadherin-positive cells in the MSC culture at the twelfth and eighteenth passages was stable and moderate, indicating the presence of cells with epithelial markers at late passages (Fig. 1k).

Cytogenetic analysis of rabbit bone marrow mesenchymal stem cells

The results of analysis of the metaphase plate of rabbit MSCs at the first, third and fifth passages after enzymatic (the first series of experiments) and chelating (second series of experiments) dissociation of cellular material are shown in Table 2 and Fig. 2. As seen in the illustrations, quantitative chromosomal changes, including aneuploidy and polyploidy, are characteristic for these cells. Aneuploidy in rabbit MSCs after enzymatic dissociation of cell material was manifested at a rate of 15% at the first, 15.4% at the third and 8.3% at the fifth passage. Cytogenetic variability was mainly manifested by the appearance of hyperploid cells with karyotype 2n = 46 or 2n = 56 in the cell cultures (Fig. 2a2).

Multiple increases in haploid sets of chromosomes in rabbit MSCs were noted following enzymatic dissociation of cell material at all passages. Polyploid cells were mainly tetra- and hexaploid with karyotype 2n = 110 and 2n = 132, respectively. The percentage of polyploid cells in rabbit mesenchymal stem cells was 6.3% at the first passage, 15.4% at the third passage, and 37.5% at the fifth passage. In populations of cells from the first to the fifth passages an increase was observed in the percentage of cells with polyploidy, with significant differences between means (r≤0.001), indicating the karyotypic instability of the cell cultures subjected to enzymatic dissociation of cell material.

Multiple increases in the number of chromosomes (polyploidy) were manifested in the cell population at the first and fifth passages following chelate dissociation of cellular material. The percentage of cells with a polyploid karyotype at the first passage was 5.5%. At the third passage no polyploid cells were found. The frequency of polyploid cells at the fifth passage was 26%. Polyploid cells were mostly tetraploid, with karyotype 2n = 88.

No structural karyotype disturbances (chromosomal breaks) in populations of rabbit MSCs were detected following enzymatic dissociation of cellular material from the first to fifth passages, whereas after chelate dissociation of cellular material chromosomal breaks were detected in cells at the first passage (5.5%). In populations of rabbit MSCs at the third and fifth passages no structural chromosome defects were found.

Thus our study has shown that enzymatic dissociation of cell material leads to much greater aberrations in the chromosome apparatus of cultivated cells than in the case of chelate dissociation of cellular material. In particular, quantitative infringement of chromosomes appears in cells cultured at the third and fifth passages. Thus, after chelate dissociation of cellular material the number of cells with polyploidy was 13% less than in the case of enzymatic dissociation at the first passage; in cells at the third passage polyploidy was not observed following chelate dissociation, whereas after enzymatic dissociation of cellular material polyploidy was 15.4% at the third passage. At the fifth passage after chelate dissociation of cell culture polyploidy was 26%, which was 1.44 times less than in cell cultures subjected to enzymatic dissociation (37.5%).

A micronucleus test was performed to evaluate karyotype destabilization of MSC (Table 3, Fig. 3). The proportion of binucleated cells at the first and fifth passages was 7.2‰ and 8.2‰ respectively.

Table 2. Indicators of cytogenetic analysis of rabbit MSCs at the early passages during cultivation in vitro following different methods of dissociation of cellular material (M ± m, n=5).

Methods of dissociation of cellular material	Aneuploidy, %	Polyploidy, %	Chromosomal breaks
I passage			
Enzymatic dissociation	15.0 ± 2.3	6.3 ± 1.2	–
Chelating dissociation	11.1 ± 2.1	5.5 ± 0.9	5.5 ± 1.2
III passage			
Enzymatic dissociation	15.4 ± 2.5	15.4 ± 1.4***	–
Chelating dissociation	11.7 ± 1.5	–	–
V passage			
Enzymatic dissociation	8.3 ± 1.1*	37.5 ± 3.2***	–
Chelating dissociation	12.0 ± 1.5	26 ± 2.0***	–

* $p<0.05$, *** $p<0.001$ compared with cells at the I passage

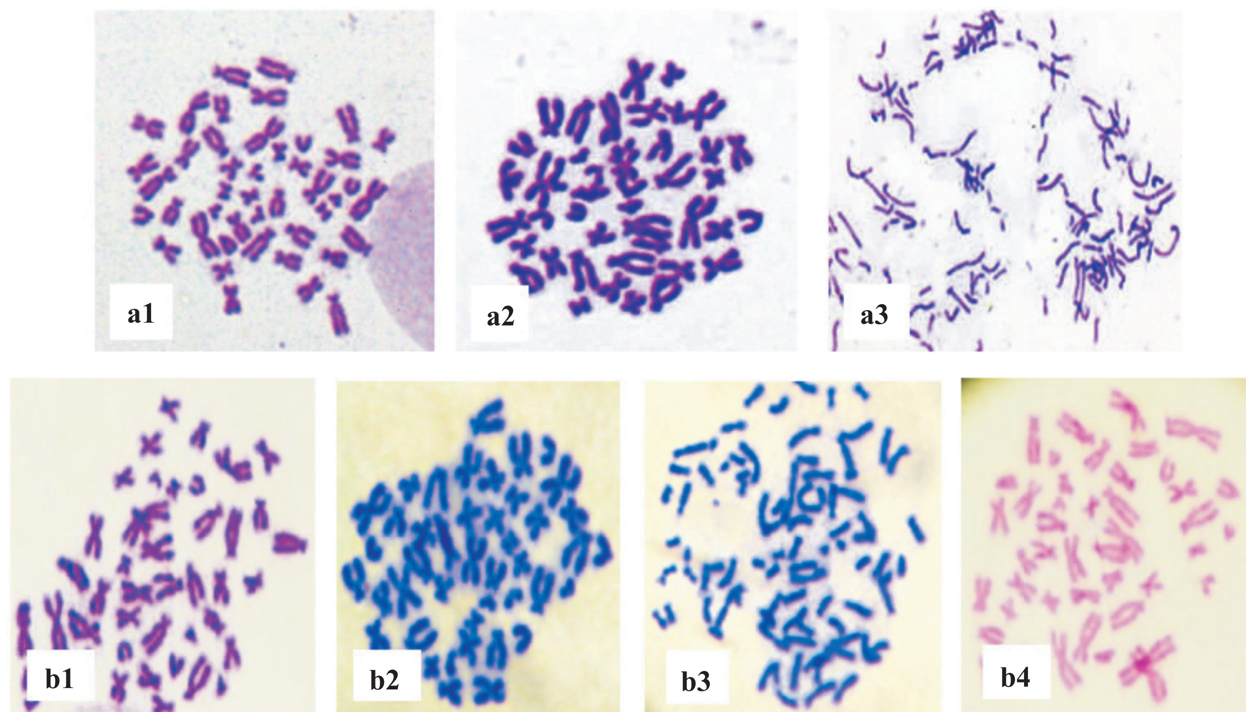

Fig. 2. Karyotype of rabbit mesenchymal stem cells (a – after enzymatic dissociation of cell material; b – after chelate dissociation of cellular material): a1 – norm (2n = 44), a2 – aneuploidy (2n = 46), a3 – polyploidy (5n = 110); b1 – norm (2n = 44), b2 – aneuploidy (2n = 42), b3 – polyploidy (2n = 88), b4 – chromosomal breaks; x1,000.

The mitotic index was 11.0-15.2‰, indicating increased proliferation of cultivated cells. The level of apoptotic cells did not exceed 2.0‰ . The results of the micronucleus test following chelate dissociation of cellular material showed that the number of cells with micronuclei in populations of rabbit mesenchymal stem cells was within 3-7‰. The number of these cells increased with each successive passage. The proportion of cells with micronuclei was 3‰ at the first passage, 5‰ at the third, and 7‰ at the fifth. The difference between the average values for this characteristic in populations of cells at the first and fifth passages was significant ($r \le 0.05$). The mitotic index of MSC subjected to chelate dissociation at the first and fifth

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Table 3. Results of micronucleus test of rabbit MSCs at early passages during cultivation following different methods of dissociation of cellular material (M ± m, n=5).

Methods of dissociation of cellular material	Cells with micronuclei, ‰	Binucleated cells, ‰	Mitotic index, ‰	Apoptotic cells ‰
I passage				
Enzymatic dissociation	9.0 ± 2.6	4.0 ± 1.1	11.0 ± 1.4	2.0 ± 1.0
Chelating dissociation	3 ± 0.9	–	3 ± 2.1	–
III passage				
Enzymatic dissociation	4.0 ± 1.7	7.2 ± 1.6	10.1 ± 1.3	1.0 ± 1.0
Chelating dissociation	5 ± 1.5	3 ± 0.7	4 ± 2.3	–
V passage				
Enzymatic dissociation	3.2 ± 1.3	8.2 ± 1.5*	15.2 ± 2.2	2.0 ± 1.0
Chelating dissociation	7 ± 1.9*	4 ± 1.8	4 ± 0.5	2 ± 0.3

** p<0.05 compared with cells at the I passage

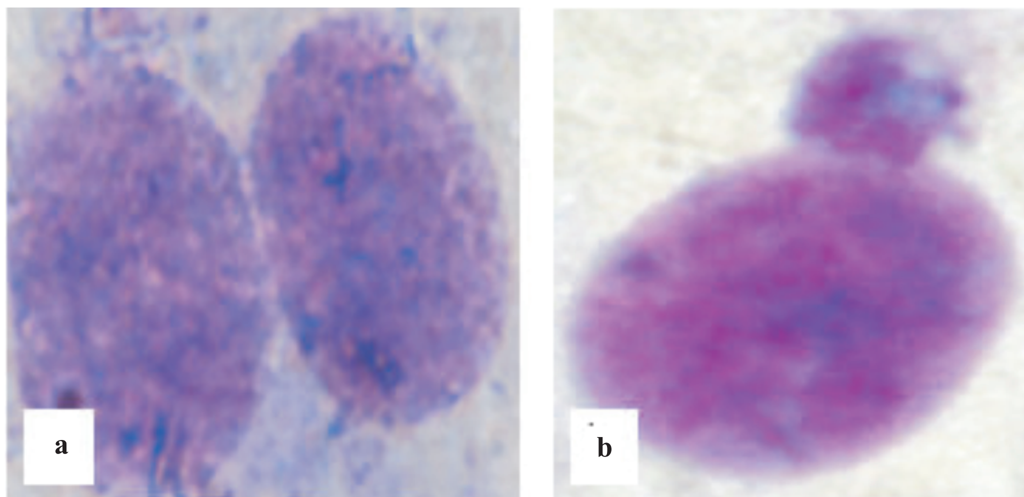

Fig. 3. Visualization of the micronucleus test conducted on rabbit MSCs (third passage) following enzymatic dissociation of cell material: a – binucleated cell; b – cells with micronuclei, x1,000.

passages was 3-4‰. Apoptosis was observed only at the fifth passage (2‰). Thus, the analysis of the micronucleus test indicated that the cell culture which was subjected to enzymatic dissociation significantly exceeded the level of spontaneous mutagenesis characteristic for mammals, in contrast with the cell culture subjected to chelate dissociation of cellular material. The proportion of cells with micronuclei at the first passage after enzymatic dissociation was 9.0‰, as compared to just 3.0‰ after chelate dissociation (Table 3). An increase was also noted in the proportion of binucleated cells following enzymatic dissociation of the cell culture as compared to chelate dissociation. After enzymatic dissociation of the cell material the proportion of binucleated cells at the third and fifth passages was 7.2-8.2‰ (Table 3), while in the cell culture subjected to chelate dissociation it was 3-4‰.

The increased mitotic index in the cell culture subjected to enzymatic dissociation (11.0-15.2‰) appears to be evidence of increased proliferation of specific clones of cultivating cells. The mitotic index in the cell culture subjected to chelate dissociation was 3-4‰. The level of apoptotic cells was in the normal range following both the enzyme and chelating methods of dissociation. Rabbit bone marrow mesenchymal stem cells at the eighteenth passage had a significantly reduced expression of nuclear proteins associated with proliferation and the cell cycle, as evidenced by the significant (r≤0.001) downregulation of RSNA (2.7 times) and Ki67 (6 times).

Discussion

Our results on the cytogenetic stability of mesenchymal stem cells from rabbit bone marrow following enzymatic and chelate dissociation are consistent with studies by a number of researchers. In particular, enzymatic dissociation of bovine primary cultures of embryonic fibroblasts has been shown to lead to an increasing number of aneuploid cells with each passage, reaching up to 85% at the later passages, while the initial level of aneuploidy in the culture was 47% (Giraldo et al. 2006). Furthermore, during cultivation of mesenchymal stem cells of pig fat tissue, aneuploidy was found to be 46.6% at the first-tenth passages, 87% at the eleventh-twentieth passages, and 94.4% at the twenty-first-thirtieth passages (Williams 2009).

The appearance of polyploid cells is linked to a decrease in the mitotic potential of cells and the loss of capability of cytoplasmic division (Matsumura 1980). The proportion of cells with micronuclei following enzymatic dissociation of cell material, where the norm is 1.6-5.6‰ for mammals, was increased (Wang et al. 2005).

The level of cytogenetic variability (percentage of cells with micronuclei) in rabbit MSCs at the fifth passage was higher than the spontaneous variability characteristic for mammals (the normal rate is 1.6-5.6‰) (Xikum and Liming 1990). The increase in the proportion of cells with each successive passage may reflect not only the intensity of the emergence of new somatic mutations but also their accumulation over time. No differences were observed between numbers of binucleated cells in populations of rabbit MSCs depending on the number of the passage.

Thus, the use of ethylenediaminetetraacetic acid for dissociation of a culture of rabbit mesenchymal stem cells is more advantageous than the enzyme method of dissociation, since it has a smaller impact on their cytogenetic variability and makes it possible to obtain a cell culture with fewer quantitative and qualitative changes in the chromosome apparatus. Chelate dissociation of cellular material leads to binding of divalent metal ions of specific cellular receptor molecules of the extracellular matrix (Ca^{2+}-dependent cadherin), which impairs the adhesive properties of cells. Chelating agents, such as EDTA, do not affect the amino acid composition of adhesive proteins. Unlike chelate dissociation, the mechanism of enzymatic dissociation of the cell monolayer is that the enzyme acts directly on integrin ligands (fibronectin, laminin and collagen), which in turn leads to disruption of intercellular contacts and their interaction with vinculin (Yamada and Geiger 1997). This can impair transmission of information to the cell nucleus and lead to the emergence of karyotypic deviations. Furthermore, changes in the karyotype may lead to cessation of cell division and accelerate cellular ageing and apoptosis (Andraszek et al. 2016).

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