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Immunophenotypic characterisation and cytogenetic analysis of mesenchymal stem cells from equine bone marrow and foal umbilical cords during *in vitro* culture

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Abstract

Introduction: The objective of the study was immunophenotypic and cytogenetic analysis of mesenchymal stem cells from equine bone marrow and foal umbilical cords during *in vitro* culture. **Material and Methods:** The mesenchymal stem cells were obtained from equine bone marrow of three horses and from foal umbilical cords of six foals. The cells were cultured in CO_2 incubators by standard procedure. Quantitative abnormalities of chromosomes, *i.e.* aneuploidy and polyploidy, and structural aberrations, *i.e.* breaks in chromosomes and chromatid, were taken into account during the study. **Results:** The results of cytogenetic analysis of equine bone marrow mesenchymal stem cells at the third and fourth passages indicated that the level of karyotype variability of these cells corresponded to the spontaneous level of karyotype variability typical of the peripheral blood lymphocytes of this species. Equine bone marrow contained several clones of stem cells that differed in the expression of specific nuclear markers characteristic of proliferating cells. **Conclusion:** Mesenchymal stem cells from foal umbilical cords during *in vitro* cultivation are characterised by quantitative abnormalities of the chromosomal apparatus.

Keywords: horses, bone marrow, umbilical cord, mesenchymal cells, cytogenetic analysis.

Introduction

The pathology of the musculoskeletal system is a common problem in the sports horse industry due to the extremely high loads on the tendons, joints, *etc.* during training or sports competitions. Conservative methods for treating horses with injured tendons are often ineffective and are associated with a high risk of retraumatisation, in about 55%–66% of horses during subsequent physical activity (11).

Techniques using stem cells occupy a leading position among the scientific advances in regenerative therapy. Mesenchymal stem cells from animal bone marrow and adipose tissues are used most frequently. In recent years, however, an increased use of stem cells from non-embryonic mammalian organs, particularly from the umbilical cord, has been observed. The umbilical cord contains a new class of pluripotent stem cells with intermediate properties between those of embryonic and somatic stem cells (8, 21). These cells

can be obtained without additional surgery or ethical constraints (15, 24). Subsequently, they can be successfully used in cell-regenerative therapy as autologous material for treatment of animals with musculoskeletal system pathology. However, an extremely important issue arising from the use of stem cells in cell-regenerative therapy is its safety, in terms of the risk of neoplastic transformation after administration *in vivo*. During *in vitro* culture mesenchymal stem cells undergo changes that result in cellular aging and reduced cell proliferation. Mutations which appear during cultivation may lead to neoplastic transformation of cells. For this reason, before being transplanted into animals, mesenchymal stem cells should be checked against the cancerous phenotype and tested for the presence of mutations that can lead to neoplastic transformation and tumour growth (7).

The literature offers conflicting data regarding the risk of spontaneous transformation of mesenchymal stem cells during *in vitro* culture (2-4, 16). Therefore, investigations of the biological properties of mesenchymal stem cells from equine bone marrow and foal umbilical cords as well as their cytogenetic analysis are highly relevant and timely tasks (13).

The objective of the study was to perform an immunophenotypic and cytogenetic analysis of mesenchymal stem cells from equine bone marrow and foal umbilical cords during *in vitro* culture.

Material and Methods

Obtaining mesenchymal stem cells from equine bone marrow. Bone marrow was collected from the iliac crest of clinically healthy one- to two-year-old horses. Three animals from the Derkulsk State Horse Stud (Novoderkul, Belovodsk district, Luhansk region) were used in the experiment. All manipulations were performed under general anaesthesia and subsequent infiltration of the soft tissues near the site of the bone puncture with 2% Lidocaine solution. The animals were sedated by intravenous injection with Detomidin (0.02 mg/kg b.w.) As a general anaesthetic, Propofol was administered intravenously at a dose of 2 mg/kg b.w.

Bone marrow was collected using a needle for the bone tissue trepanobiopsy (Sterylab, Italy). Before bone aspiration, the syringe was filled with heparin at 2–3 IU per 1 mL of its volume. After bone marrow aspiration, the syringe was disconnected from the needle, and the needle was removed by turning it anticlockwise. To stop bleeding, a sterile cotton-gauze swab was applied to the site of the skin puncture for a few minutes and then the puncture site was treated with a 5% alcoholic iodine solution.

To obtain the mononuclear cell fraction, the bone marrow aspirate was diluted with phosphate buffer solution at a ratio of 1:5 and centrifuged in a gradient density of Ficoll (ρ = 1.076) for 30 min at 300 g. Mononuclear cells in the amount of 625 000/cm^2 were transferred to Petri dishes (d = 35 mm) for culturing.

Obtaining mesenchymal cells from foal umbilical cords. Umbilical cords were obtained from foals of thoroughbred horses during birth (Millennium, Kliuchove village, Donetsk region, Volodarskii district).

Samples of the umbilical cords were obtained immediately after birth (six foals were used in the experiment). The cords were washed in tap water to fully remove foreign particles and transferred to a bowl with 70% ethanol for 5 min. Then they were placed in a container filled with normal saline solution containing antibiotic and antimycotic agents, transferred to a transport container (4ºC), and transported to the laboratory. Further procedures were carried out in a sterile laminar hood. The umbilical cords were washed several times with PBS, released from the vessels, and cut into pieces of 3–5 mm. Then the tissues were exposed to enzymatic disaggregation with 0.25% trypsin solution at 4ºC for 36 h. The cell suspension obtained was filtered through four layers of sterile gauze cloth, centrifuged, resuspended in culture media, and seeded in Petri dishes.

Cells cultivation. The cells from the equine bone marrow and umbilical cords were seeded in Petri dishes (d = 35, 60 mm) and cultured in CO_2 incubators (5% CO_2 and 37.0ºC) by standard procedure. The culture media contained 80% Dulbecco's modified Eagle's medium and 20% foetal bovine serum with 10 µl/mL of antibiotic-antimycotic solution. The culture medium was replaced every 72 h. When monolayer confluency reached about 80%–90%, the cells were transferred to a suspension using 0.05% trypsin-EDTA solution and reseeded in 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.

Immunophenotypic 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 20ºC, 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 (clon CD 325, ThermoScientific), vimentin (V9, DiagnosticBioSystems), and CD24 (SN3b, NeoMarkers) for 30–60 min in accordance with the instructions for monoclonal antibody application. Then the PolyVue visualisation system (ThermoScientific) conjugated with peroxidase was used and enzyme activity was detected using diaminobenzidine (ThermoScientific) as a substrate. After conducting an immunocytochemical reaction, the preparations were washed with water and stained with Mayer

haematoxylin (Sigma) for 15–30 s, and then placed in Faramount Aqueous Mounting Medium. The results were analysed by counting the number of positively stained cells (brown staining) and evaluated by the classical 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. Cytogenetic analyses were performed on cultures of mesenchymal stem cells from equine bone marrow and umbilical cords of thoroughbred horses. Thirty metaphase plates obtained by a modified standard cytogenetic method were analysed in a cell culture of each passage investigated (18). Fixation of chromosomes was performed for 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 37ºC. The cells were removed from the Petri dishes and a cell suspension was obtained by incubation for 1–5 min at 37ºC in trypsin-EDTA solution. The cells were maintained in metaphase by incubation for 30 min at 37ºC in a warm hypotonic solution of KCl (0.56%) at a rate of 1 mL of cell suspension to 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 material obtained was 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 1000×.

Quantitative abnormalities of chromosomes, *i.e.* aneuploidy and polyploidy, and structural aberrations, *i.e.* breaks in chromosomes and chromatid, were examined. A micronucleus test was performed on the material to estimate the number of binucleated cells (BC) and cells with micronuclei (CM), the mitotic index (MI), and the number of apoptotic cells (AC). The frequency of BC, CM, MI, and AC was calculated in 1000 cells (‰).

Statistical analysis. The results were statistically analysed using Student's *t*-test for significance of differences between means. Differences at $P \leq 0.01$, $P \leq 0.05$, and $P \leq 0.001$ were considered significant or highly significant. The tables show the mean and standard deviation.

Results

Morphological features of cells of mesenchymal origin during *in vitro* culture. The main difficulty in obtaining mesenchymal stem cells (MSCs) from bone marrow is associated with the presence of several cell populations that are able to bind to the plastic culture dish. For this reason the primary cell cultures from the bone marrow were characterised by a considerable morphological heterogeneity until the 1st and 2nd passage. In this case, two types of cells predominated: round cells that grew in colonies and divided intensively over the first 9–13 d of *in vitro* cultivation (evidently endothelial precursors) and fibroblast-like cells. At the 2nd passage the cell culture appeared completely homogeneous and contained actively proliferating fibroblast-like cells (Fig. 1. a1-a2).

The MSCs obtained from the umbilical cords and the primary MSCs from the bone marrow were characterised by a considerable morphological heterogeneity. However, during the cell adaptation at the 0th and 1st passages, the fibroblast-like cells of the umbilical cord (Fig. 1. b1-b2, c1-c2) were morphologically similar to the fibroblast-like cells from the bone marrow, and had a flattened shape and the same size in the longitudinal and transverse directions.

The Fig. 1. b1-b2 was obtained by photographing the cultures under a microscope during days 10-15 of the cultures. The primary culture of MSCs from the equine umbilical cord had a considerable morphological heterogeneity: along with fibroblast-like cells, round cells, which were clearly endothelial cells, were observed. However, at the 0th passage, with the confluence of monolayer cells increasing to 70%–80%, the round cells were replaced by fibroblast-like cells. At the 2nd, 3rd, and consecutive passages of cultivation, the growth of round cells was not observed.

When 85% confluence was attained, in all cases, irrespective of the passage number, there was a significant decline in the mitotic activity of cellular elements of MSCs from the umbilical cord (Fig. 1. b1-b2, c1-c2).

After the 2nd passage the cultures of MSCs from both sources were morphologically homogeneous and contained mostly small, active spindle cells (Fig. 1. a1, c2). After the 5th passage, the culture of equine bone marrow stem cells and after the 7th passage the culture of umbilical cord stem cells gradually lost their mitotic activity, and some cells became large and flattened.

At this stage, the growth of the cell population had almost stopped.

Phenotypic characterisation of equine bone marrow mesenchymal stem cells. Immuno-cytochemical analysis of the CD-receptor system of equine bone marrow stem cells at the early passages indicates that the set of specific proteins differs significantly in various clones of cultured cells and changes during cultivation. The data pertaining to the immunophenotypic profile of multipotent stem cells from equine bone marrow at the 2nd and 5th passages are given in Table 1 and Fig. 2. f-h.

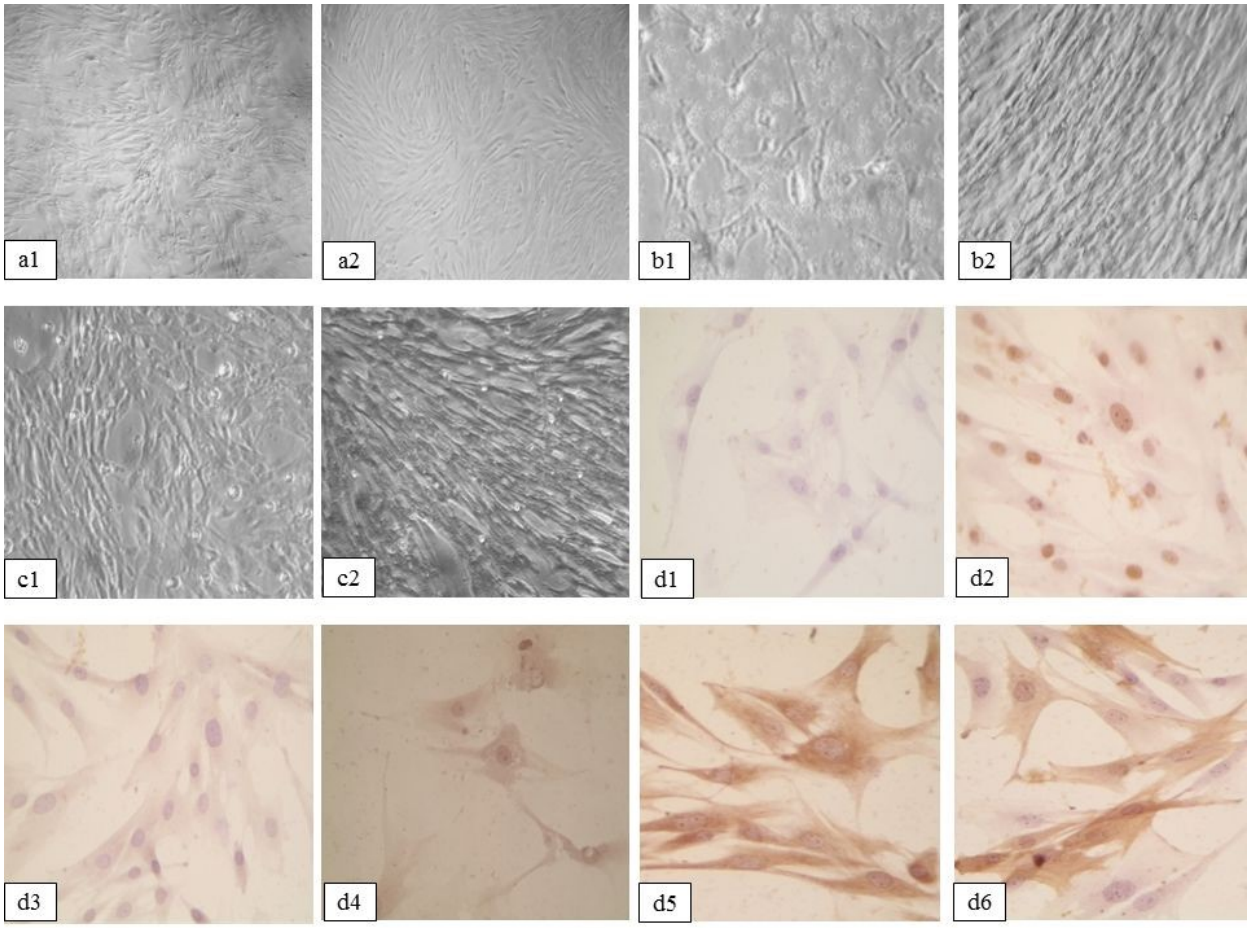

Fig. 1. A – native unstained culture of equine bone marrow mesenchymal stem cells: a1 – 2th passage; a2 – 5th passage, 100×; B – native unstained MSC culture. Formation of monolayer of primary MSC culture from foal umbilical cord (b1 – 10 d of cultivation, b2 – 15 d of cultivation), 100×; C – native unstained culture of foal umbilical cord mesenchymal stem cells: c1 – 1st passage, c2 – 5th passage, 100×; D – immunophenotypic characterisation of equine bone marrow multipotent stem cells: d1 – control, d2 – proliferating cell nuclear antigen-positive cells; d3 – Ki-67-negative cells (5th passage); d4 – Ki-67 - positive cells (2nd passage), 400×; d5 – vimentin-positive cells (5th passage); d6 – actin-positive cells, (5th passage), 400×

Table 1. Immunophenotypic profile of equine bone marrow multipotent stem cells at the early passages (mean ±standard deviation, n = 6)

Antigen	Passage of cells from equine bone marrow *in vitro*	
	2nd	5th
	Assessment in points by the H-Score method (from 0 to 300)	
	Nuclear proteins related to proliferation and cell cycle	
PCNA	0	242 ± 22
Ki-67	142 ± 11	0
	Proteins of cell adhesion and cytoskeleton	
Vimentin	229 ± 21	274 ± 11**
Actin	128 ± 11	221 ± 27*
E-cadherin	138 ± 12	0
N-cadherin	109 ± 18	0
CD24	94 ± 6	0
CD44	46 ± 9	0

* P < 0.01, ** P < 0.05

During the immunophenotypic characterisation of equine bone marrow multipotent stem cells, special attention was paid to nuclear proteins associated with the proliferation and cell cycle.

The immunocytochemical analysis showed that the number of PCNA-positive cells from the equine bone marrow was significantly different at the 2nd and 5th passages (Table 1, Fig. 1. d1-d4). At the 2nd passage, PCNA-positive cells were not detected, while at the 5th passage an increase in expression to 242 points was observed. It should be noted that the expression of another protein that characterises the proliferative potential of Ki-67 was moderate (142 points) at the 2nd passage while at the 5th passage positive cells expressing this protein were not identified.

A specific marker of mesenchymal cells is vimentin, a protein of intermediate filaments of the cell cytoskeleton. We found a considerable number of positive cells with high activity of vimentin expression at the 2nd passage (229 points) and a nearly 20% increase in its activity at the 5th passage (274 points), indicating that the cells had mesenchymal origin (Table 1, Fig. 1. a1). Immunophenotyping of equine MSCs revealed a moderate number of actin-positive cells at the 2nd passage (128 points) and a relatively large number of actin-positive cells at the 5th passage (221 points), which also points toward their mesenchymal origin. At the same time, we observed an interesting fact of clonal specificity – high intensity of actin expression in certain cell populations (Fig. 1. d5-d6).

When characterising equine bone marrow multipotent stem cells at the early passages, special attention was paid to cadherins – proteins responsible for Ca^{2+}-dependent intercellular interactions, particularly during embryogenesis and tissue differentiation, including E-cadherin, which is typical of epithelial cells in adults, and N-cadherin, which is mainly found on the surface of nerve and muscle cells. The number of E-cadherin- and N-cadherin-positive cells at the 2nd passage was 138 and 109 points, respectively, whereas at the 5th passage these cells were not detected (Table 1, Fig. 2. a-d).

During immunophenotyping of equine MSCs after expansion *in vitro*, we found a small number of CD24-positive cells at the 2nd passage and the complete absence of expression of this protein at the 5th passage (Table 1, Fig. 2. e).

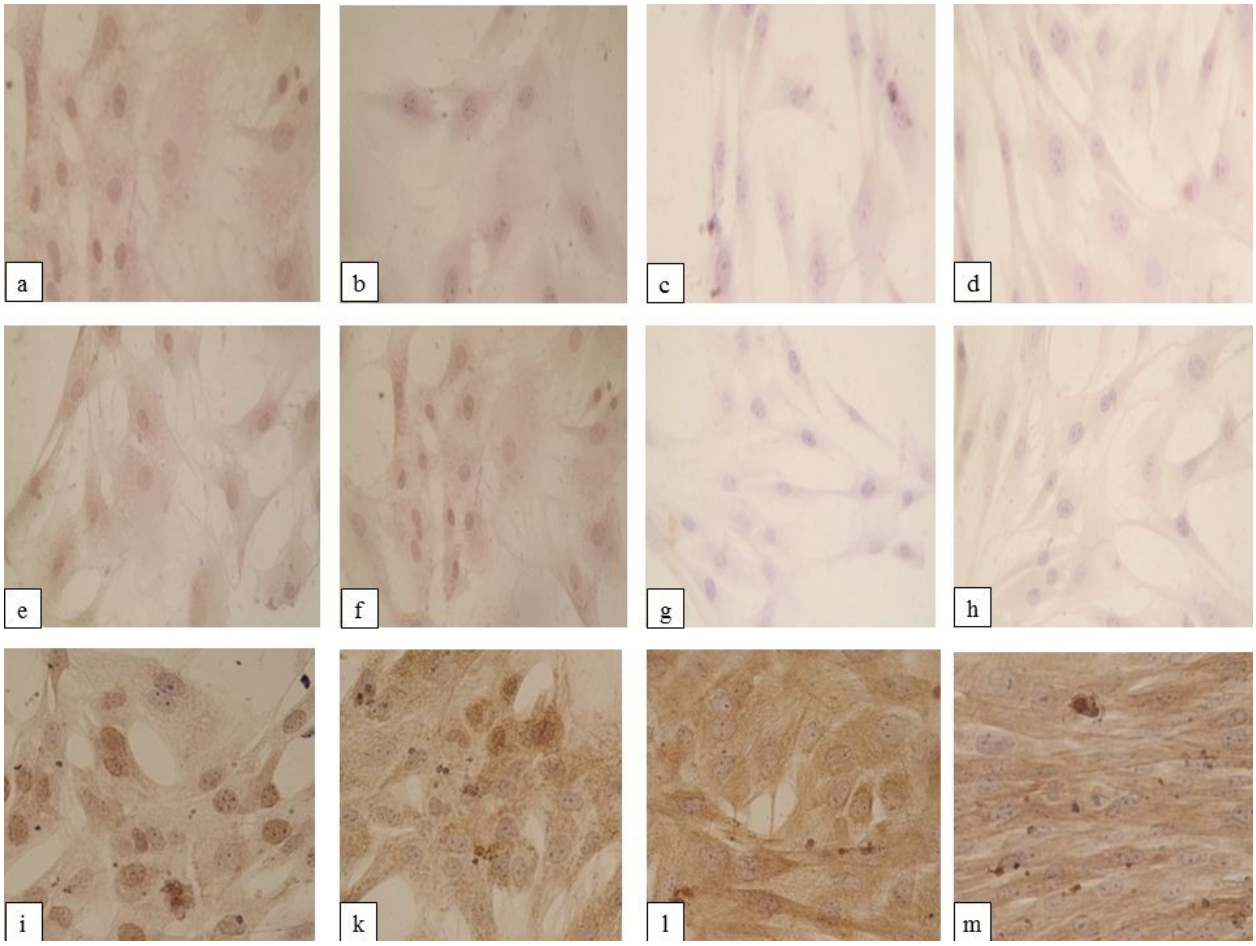

Fig. 2. Immunophenotypic characterisation of equine bone marrow multipotent stem cells: a – N-cadherin-positive cells; b – E-cadherin-positive cells (2nd passage); c – N-cadherin-negative cells; d – E-cadherin-negative cells (5th passage); e – CD24-cadherin-positive cells; f – CD44-cadherin-positive cells (2nd passage); g – CD24-cadherin-negative cells; h – CD44-cadherin-negative cells (5th passage), 400×. Immunophenotypic characterisation of multipotent stem cells from foal umbilical cords (2nd passage): i – PCNA-positive cells; k – Ki-67-positive cells; l – vimentin-positive cells; m – actin-positive cells, 400×

Investigation of cell adhesion protein CD44, which is the main cell membrane receptor for hyaluronate and is actively involved in the formation of physical contact between stromal cells and early precursors of B-cells, revealed a small number of CD44-positive cells at the 2nd passage and, as in the previous case, the complete absence of CD44 expression at the 5th passage.

Thus, the immunophenotypic heterogeneity of equine bone marrow MSCs decreased during the cultivation process. At the 5th passage in selective medium with foetal bovine serum, the equine bone marrow multipotent stem cells displayed morphological and phenotypic homogeneity and did not contain cells expressing endothelial or haematopoietic markers.

Phenotypic characterisation of mesenchymal stem cells from foal umbilical cords. Immunocytochemical analysis of the CD-receptor system of mesenchymal stem cells from foal umbilical cords at the 2nd passage indicates a selective dominance of mesenchymal cells over cells with epithelial and haematopoietic characteristics. The data pertaining to the immunophenotypic profile of multipotent stem cells from foal umbilical cords at the 2nd passage are given in Table 2 and documented photographically (Fig. 2. i-m).

In the characterisation of mesenchymal stem cells from foal umbilical cords particular attention was paid to cadherins. The number of E- and N-cadherin-positive cells was low, indicating very low expression of proteins that are typical for epithelial, neural, and skeletal cells of animals (Table 2). The small number of E- and N-cadherin-positive cells at the 2nd passage demonstrates the dominance of cells with mesenchymal characteristics over cells with epithelial characteristics.

Immunophenotyping of mesenchymal stem cells from foal umbilical cords at the 2nd passage did not detect CD24- and CD44-positive cells, which indicates the absence of haematopoietic marker expression by these cells.

Cytogenetic analysis of equine bone marrow mesenchymal stem cells. To establish the stability of mesenchymal stem cells, a comparative karyotype analysis of chromosomal variability of cells at the 3rd and 4th passages (Fig. 3. a-c) was conducted and compared with the level of spontaneous chromosomal variability of equine peripheral blood lymphocytes.

The results of the cytogenetic analysis of mesenchymal stem cells showed that the cells at the 3rd and 4th passages were characterised by quantitative karyotype disturbances, including aneuploidy, which accounted for 1.4% and 1.2% respectively (Fig. 3). No significant differences were found between quantitative disorders of chromosomes in the cells at different passages. No metaphase plates with polyploidy were detected in mesenchymal stem cells at the 3rd and 4th passages. Structural defects (chromosomal and chromatid ruptures) were not found in these cells either.

The frequency of equine mesenchymal stem cells with micronuclei at the 3rd and 4th passages was 1.3‰ and 0.8‰ respectively, not exceeding the level of spontaneous appearance of micronuclei in equine peripheral blood lymphocytes (Table 3).

The frequency of binucleated mesenchymal stem cells at the 3rd and 4th passages was 1‰ and 1.5‰, being within the parameters typical for mammalian spontaneous somatic mutagenesis.

Cytogenetic analysis of mesenchymal stem cells from foal umbilical cords. The results pertaining to the karyotype stability of mesenchymal stem cells from the foal umbilical cords according to the comparative analysis of chromosomal variability of cells at the 2nd, 3rd, 4th, 5th, and 7th passages are shown in Fig. 3. d-g. A comparison was made with the level of spontaneous chromosomal variability of equine peripheral blood lymphocytes and mesenchymal stem cells from the bone marrow of these animals. The results of the micronucleus test of these cells are presented in Table 4 and Fig. 4. a-b.

The results of the cytogenetic analysis of mesenchymal stem cells from the foal umbilical cords showed that these cells had typical quantitative chromosome abnormalities (aneuploidy and polyploidy). The percentage of metaphase plates with aneuploidy in the cells from the 2nd to 7th passages was 16.7%–40.6%, which is significantly higher than the spontaneous level of chromosome variability in equine peripheral blood lymphocytes (1.98%–6.8%) and mesenchymal stem cells from bone marrow (1.2%–1.4%). An increase in the percentage of aneuploidy was observed in cell populations from the 2nd to 7th passages, which indicates an increase in the karyotype instability of these cells with each successive passage.

The mesenchymal stem cells from the foal umbilical cords were characterised by the presence of a tetraploid karyotype (4 n = 128), which was present in cell populations at the 2nd, 3rd, and 5th passages and constituted 25%, 27%, and 4.8% respectively. The percentage of polyploid cells at the 2nd and 3rd passages exceeded the level of spontaneous chromosomal variability of equine peripheral blood lymphocytes more than five times (0.4%–4.7%).

No structural disorders of chromosomes (chromosomal or chromatid ruptures) were observed in the cell populations studied.

The results of the micronucleus test indicate that the frequency of cells with micronuclei was two-five times higher than the level of peripheral blood lymphocytes with micronuclei (1.53‰) (Table 4). An increase in the number of mesenchymal stem cells with micronuclei was accompanied by a simultaneous increase in the number of cells with aneuploidy.

Table 2. Immunophenotypic profile of multipotent stem cells from foal umbilical cords at the 2nd passage (mean ±standard deviation, n = 6)

Antigen	2nd passage Assessment in points by the H-score method (from 0 to 300)
	Nuclear proteins related to proliferation and cell cycle
PCNA	198 ± 13
Ki-67	134 ± 23
	Proteins of cell adhesion and cytoskeleton
Vimentin	265 ± 12
Actin	244 ± 19
E-cadherin	11 ± 5
N-cadherin	64 ± 9
CD24	0
CD44	0

Table 3. Results of micronucleus test of mesenchymal stem cells from equine bone marrow. Frequency in 1000 cells (‰)

Passage	Cells with micronuclei	Binucleated cells	Mitotic index	Apoptosis
3	1.3	1.0	3.0	1
4	0.8	1.5	3.3	1

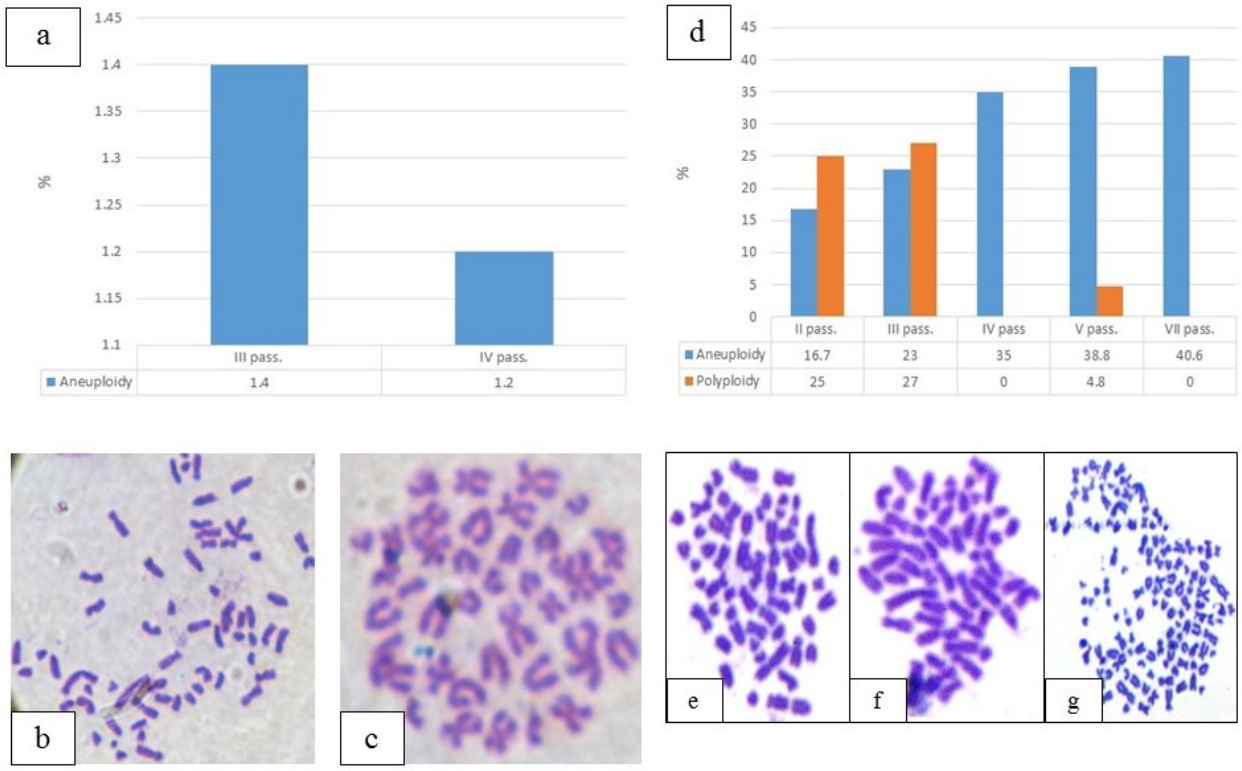

Fig. 3. Cytogenetic analysis of equine bone marrow mesenchymal stem cells: a - analysis of karyotype stability of equine bone marrow mesenchymal stem cells at the 3rd and 4th passages. Karyotype of mesenchymal stem cells of equine bone marrow mesenchymal stem cells (5th passage); b – normal (2n = 64); c – aneuploidy (2n = 59), 1,000×; d - quantitative chromosome abnormalities of mesenchymal stem cells from foal umbilical cords at the early passages. Karyotype of mesenchymal stem cells from foal umbilical cords (5th passage): e – normal (2n = 64); f – aneuploidy (2n = 54); g – polyploidy (4n = 128), 1,000×

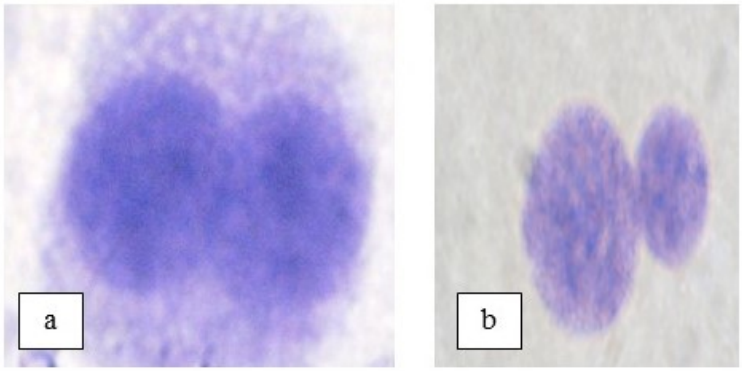

Fig. 4. Micronucleus test of mesenchymal stem cells from foal umbilical cords: a – binucleated cell; b – cell with micronuclei, 1000×

Table 4. Results of the micronucleus test performed on mesenchymal stem cells from foal umbilical cords. Frequency in 1000 cells (‰)

Passage	Cells with micronuclei	Binucleated cells	Mitotic index	Apoptosis
2	1.3 ± 0.23	3.7 ± 0.75	4.0 ± 0.5	-
3	4.0 ± 0.39*	1.0 ± 0.3**	6.0 ± 0.7	-
4	3.0 ± 0.9	2.0 ± 0.6	4.0 ± 1.0	-
5	5.7 ± 1.1*	2.3 ± 0.5	2.7 ± 0.5	0.7 ± 0.2
6	8.0 ± 1.2***	0.3 ± 0.2*	6.0 ± 0.9	1.3 ± 0.4

* P < 0.01, ** P < 0.05, *** P < 0.001. Comparisons are made between passage 2 and others

Discussion

The immunocytochemical analysis showed that the number of PCNA-positive cells from the equine bone marrow was not significantly different at the 2nd and 5th passage. Obviously, equine bone marrow contains several clones of multipotent stem cells, which differ in the expression of specific nuclear markers that are present in the proliferating cells. Our findings are in agreement with the results obtained by other researchers (9). The mesenchymal stem cells from umbilical cords expressed specific nuclear markers which are inherent to proliferating cells. This confirms the mesenchymal nature of the cells investigated. Our results are in agreement with the studies conducted by a number of researchers (10, 12). The fraction of mesenchymal stem cells with aneuploidy did not exceed the level of spontaneous chromosomal variability in equine peripheral blood lymphocytes (6, 17). For a more complete assessment of somatic mutagenesis of mesenchymal stem cells, a micronucleus test was performed. The formation of cells with micronuclei is the result of chromosomal ruptures or defects in the mitotic spindle, which is consistent with the manifestation of aneuploidy (15, 18, 19, 22-23). The normal frequency of cells with micronuclei in mammals is 1.6‰–5.6‰ (8). Thus, the fraction of cells with micronuclei was within the normal range.

The presence of binucleated cells results from cell aging and natural extension of the duration of cytokinesis (20). The frequency of appearance of binucleated cells was in direct proportion to the mitotic index of these cells. The level of apoptotic cells at the 3rd and 4th passages in horses did not exceed the parameters typical for this species (6). It should be noted that the chromosome materials obtained by modified standard cytogenetic methods did not show increased levels of disruption of the cytoplasmic membrane integrity of MSCs compared to peripheral blood lymphocytes (19).

According to published data, the presence of a tetraploid karyotype leads to the cessation of division, acceleration of cellular aging, and apoptosis (1). Changes in the amount of genetic material during cultivation can lead to abnormal functioning of the cell genome and negative effects in the cell, including oncogenic transformation (5).

The mitotic activity of the investigated cells increased as well. The level of apoptotic cells at the 5th and 7th passages did not exceed the parameters characteristic for this animal species. The frequency of appearance of binucleated mesenchymal stem cells from the umbilical cords at the 2nd, 4th, and 5th passages exceeded the parameters characteristic of mammalian cells with spontaneous somatic mutagenesis (1.13‰), indicating destabilisation of the cell karyotype.

In conclusion, equine bone marrow contains several clones of stem cells that differ in the expression of specific nuclear markers which are typical of proliferating cells. Mesenchymal stem cells from equine bone marrow at the 5th passage are morphologically and phenotypically homogeneous and do not contain cells that express endothelial and

haematopoietic markers. Stem cells from foal umbilical cords express markers which are typical of cells of mesenchymal origin and do not express haematopoietic markers. The level of karyotype variability of mesenchymal stem cells from equine bone marrow at the 3rd and 4th passages during *in vitro* cultivation corresponds to the spontaneous level typical for this species. Mesenchymal stem cells from foal umbilical cords during *in vitro* cultivation are characterised by quantitative abnormalities of the chromosomal apparatus. We established that the limits of micronucleus test parameters for umbilical cord mesenchymal stem cells increased in comparison with mammalian peripheral blood lymphocytes during spontaneous somatic mutagenesis.

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Animal Rights Statement: The experiment was approved by the Commission on Bioethics Institute of Veterinary Medicine NAAS of Ukraine, Resolution No. 1/2012 for conduction of experiments on animals.

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