AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 6, pp. 1088-1099
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)

ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

in vitro Cell cultures

UDC 619:57.085.23

doi: 10.15389/agrobiology.2023.6.1112eng
doi: 10.15389/agrobiology.2023.6.1112rus

EXTRACELLULAR VESICLES INCLUDING EXOSOMES FROM ANIMAL MESENCHYMAL STEM/STROMAL CELLS

I.P. SAVCHENKOVA ✉, G.A. NADTOCHEY

Federal Science Center Skryabin and Kovalenko All-Russian Research Institute of Experimental Veterinary RAS, 24/1, Ryazanskii pr., Moscow, 109428 Russia, e-mail s-ip@mail.ru (✉ corresponding author), g_a_nadtochei@mail.ru
ORCID:
Savchenkov I.P. orcid.org/0000-0003-3560-5045   Nadtochey G.A. orcid.org/0000-0003-4295-3400
The authors declare no conflict of interests
Acknowledgements:
The work was carried out within the framework of research project FGUG 2022-0010 “Maintenance and development of collections of cell cultures and microorganisms based on fundamental research, creation of strains of bacteria and viruses with specified properties for veterinary medicine using biotechnology methods, including those based on cellular nanobiotechnologies, improving diagnostics and means of specific prevention of infectious diseases"
Final revision received April 10, 2023 g
Accepted June 13, 2023

Abstract

Mammalian mesenchymal stem/stromal cells (MSCs) produce extracellular vesicles (EVs) associated with the plasma cell membrane, which may contain growth factors, chemokines, cytokines, and microRNAs. Currently, EVs are widely used to develop new regenerative strategies in the treatment of numerous diseases, since they convey most of the therapeutic properties of MSCs. This work shows for the first time that EVs enriched with exosomes can be isolated from conditioned media (CM) of MSCs from five different animal species using the method of differential centrifugation (DC) followed by ultracentrifugation (UC). The purpose of the work is to obtain EVs from conditioned media (CM) of MSCs of bone marrow (BM), umbilical cord blood (UCB) and adipose tissue (AT) of agricultural (cattle, sheep, horses) and small domestic animals (dogs, cats). We used MSCs that were previously obtained from the bovine and ovine BM, equine UCB, ovine, bovine, equine, canine and feline AT. MSCs were thawed and seeded into 25 cm^2 growth area flasks, after 48 hours they were reseeded into 175 cm^2 growth area flasks in a ratio 1:7 and incubated for 10 days until the cells reached a complete monolayer. Then the CM from all MSC samples was poured into 50 ml sterile centrifuge tubes and EVs were isolated. For this purpose, we used the method of DC followed by UC. In all samples, electron microscopy revealed round or irregularly shaped microparticles of different sizes. The diameters of individual EVs did not differ statistically between different animal species ($p = 0.1$). When comparing the number of particles isolated from 50 ml of CM from MSCs of different animal species, no statistically significant differences were detected ($p = 0.1$). Thus, on one mesh of bovine MSC(BM) and MSC(AT), 3±0.1 and 6±0.07 particles with a size of 50-100 nm, 7±0.02 and 4±0.03 particles of 100- 150 nm, as well as 3±0.4 and 2±0.06 particles larger than 150 nm. EVs from CM of canine MSC(AT) were the most homogeneous in both shape (round) and size, and the main part was found in the range of 50-100 nm (12±0.02 particles). In samples isolated from the CM of ovine, bovine, equine MSC(AT) and equine MSC(UCB), the number of particles with a diameter of 50-100 nm was 7±0.2; 7±0.01; 5±0.7 and 8±0.02. Analysis of the obtained electron diffraction patterns showed that more than 70 % of EVs had a diameter from 50 to 100 nm, that is, they were classified as exosomes. EVs isolated from CM canine MSC were positively stained with antibodies against the TSG101 antigen (cytoplasmic protein, exosome marker). The results obtained demonstrate that the CM of animals MSCs, isolated from BM, AT and UCB contains EVs, including exosomes. The DC method does not exclude the possibility that other particles are present in the preparation, so we propose to designate the result obtained as micro explosives enriched with exosomes. Obtaining EVs of a certain composition from the CM of agricultural and domestic animal MSCs opens up broad prospects for the use of exosomes in the diagnosis of diseases and treatment of agricultural and domestic animals.

Keywords: mesenchymal stem/stromal cells, bone marrow, adipose tissue, umbilical cord blood, horses, cattle, sheep, dogs, cats, extracellular vesicular, exosomes, isolation, differential centrifugation, identification

Animal мesenchymal stem/stromal cells (MSCs) have been attracting

attention for several decades as a promising cellular material in regenerative medicine due to their in vitro adaptation and proliferation, differentiation ability, and powerful immunomodulatory and anti-inflammatory properties [1, 2]. It is possible that the regenerative abilities of MSCs are due to pleiotropic effects mediated by the secretion of soluble paracrine factors and extracellular vesicles (EVs) [3, 4]. EVs are naturally produced nanoparticles. They are an important component of the secretomes of various cells, provide complex transfer of biologically active molecules and horizontal transfer of genetic information [5, 6]. EVs are present in all biological fluids of the body, consist of double layers of phospholipids and differ in origin, composition and functions. Depending on how extracellular vesicles are formed and their size, they are called apoptotic bodies, ectosomes, or exosomes.

Apoptotic bodies, also known as apothecs, are cell fragments bounded by the plasma membrane that are formed as a result of apoptosis. They are approximately 50-5000 nm in diameter and may contain organelles or even fragments of the nucleus of a dead cell. Ectosomes, or budding microvesicles, are formed by protrusion of the plasma membrane from the cell outward; the bulge is detached from the cell membrane and turns into vesicles ranging from 50 to 200 nmm sometimes up to 1000 nm, in diameter. Exosomes are small vesicles, usually 40 to 100 nm in size that appear inside the cell and bud into a cavity called an endosome. When a sufficiently large number of exosomes accumulate in this cavity, its further fate depends on what lipids its membrane consists of. If the endosome is labeled with lysobisphosphatidylic acid (phosphatidylinositol-3-phosphate) and contains ubiquitinated proteins, its contents will be destroyed, it will fuse with the lysosome, a membrane vesicle filled with enzymes that break down proteins, carbohydrates, lipids and nucleic acids. If the endosome membrane contains ceramides, the endosome fuses with the cell surface membrane, and many exosomes are pushed out into the extracellular environment [7].

Exosomes secreted by MSCs are of special interest [3, 8]. Currently, they are widely used to develop new regenerative strategies in the treatment of numerous diseases, since they convey most of the therapeutic properties of these cells. The use of MSC-derived exosomes serves as an alternative to cell therapy and may minimize safety concerns when administering viable cells [9].

Exosome function is easily regulated by pretreatment of MSC culture, for example by adding chemical factors or cytokines, creating hypoxic conditions, and introducing gene modifications [10]. However, the mechanisms of the regenerative abilities of MSC exosomes in target cells are still not well understood. In addition, there are several unresolved issues, namely the lack of standards and guidelines regarding the size of EVs, their purity, the expression of certain biomarkers (e.g., CD9, CD63, CD81) on their surface and acceptable levels of contamination for identification and quality control of isolated exosomes [11]. The use of MSC exosomes in clinical settings is also limited due to the lack of standardized cell culture conditions and optimal uniform protocols for isolation and storage of exosomes, optimal therapeutic dose and administration schedule, and reliable assays to evaluate the effectiveness of EVs therapy.

Improving methods for obtaining EVs of a certain composition opens up broad prospects for their use in the clinical practice of veterinary medicine [12]. Infectious diseases and zoonoses are among the main problems in keeping farm animals. Modern understanding of the biology of infectious agents is critical to ensuring the health and welfare of animals, maintaining their high productivity performance and food safety. It is believed that human EVs can carry viral elements, participating in both the spread of infectious diseases and the induction of an immune response against the infectious agent, and represent promising material

as diagnostic biomarkers for a better understanding of the disease pathogenic mechanisms [13]. EVs that are released by HeLa cells infected with Newcastle disease virus (NDV) or Madin-Darby bovine kidney cells (MDBK) infected with caprine parainfluenza virus type 3 (CPV3) carry microRNA, RNA or proteins that enhance the cytopathic activity of the virus. In addition, EVs were able to suppress the expression of the interferon gene (*IFN-β*) in HeLa cells after NDV infection and inhibited autophagy during CPIV3 infections, suggesting a significant role for EVs in viral spread [14, 15]. EVs are secreted by various cells and, upon adaptation to in vitro conditions, are released into the growth medium, reaching a maximum amount when the medium becomes conditioned.

This work shows for the first time that using differential centrifugation (DC) followed by ultracentrifugation (UC), EVs enriched in exosomes can be isolated from conditioned media (CM) of MSCs from five animal species.

The purpose of the work is to obtain extracellular vesicles from conditioned media of mesenchymal stem/stromal cells of bone marrow, umbilical cord blood and adipose tissue of cattle, sheep, horses and small domestic animals (dogs and cats).

Magterials and methods. MSCs from the bone marrow (BM) of cattle and sheep, umbilical cord blood (UC) of horses, adipose tissue (AT) of sheep, cattle, horses, dogs and cats were isolated by us previously [16-19] and deposited in the Specialized Collection of Continuous Transplantable Somatic Cultures of Agricultural and Game Animals RKKK (P) (SKhZh RAS, FSC ARRIEVM RAS) after identification and standardization. In the experiment of 2023, primary MSCs were thawed and plated into flasks with 25 cm^2 avaible growth area; after 48 h culture, they were re-plated (1:7) into flasks with 175 cm^2 growth area and cultured for 10 days.

The main culture medium for MSCs was DMEM with a low glucose content (1 g/l) (PanEco, Russia) supplemented with 10% bovin fetal blood serum (FBS) (HyClone, USA) and 1× solution of essential amino acids and antibiotics (PanEco, Russia). The final concentration of streptomycin was 50 μg/ml, penicillin 50 U/ml. Before use, FBS was preliminarily purified from exosomes (a L7 Ultracentrifuge, Beckman, USA; 100,000 g for 60 min).

Conditioned media (CM) were collected from MSCs cultures after they reached 100% monolayer in flasks with 175 cm^2 surface area, the CM volume per flask was 50 ml. Morphological analysis of cells was performed visually (a phase-contrast microscope with AxioVision Rel software for measurements. 4.8, Carl Zeiss, Germany).

EVs were isolated by differential centrifugation (DC). First, CM were collected in 50 ml sterile tubes (SPL Life Sciences Co, Korea) and subjected to low-speed centrifugation (500 g, 20 min, a CM-6M centrifuge, SIA ELMI, Latvia) to remove dead cells, decayed cell components and other extracellular matrix particles in the CM that sediment. The supernatant was poured into another sterile tube and filtered (filter pore size 0.22 μm; GE Osmonics, USA). The filtered CM was again centrifuged (CM-6M, SIA ELMI, Latvia) at 2300 g for 30 min to remove apoptotic bodies from the sediment, and the supernatant was subjected to high-speed centrifugation at 13000 g for 30 min (an Eppendorf 5804, Eppendorf, Germany). Finally, EVs were recovered by ultracentrifugation at 100,000 g for 60 min (a L7 Ultracentrifuge, Beckman, USA). The supernatant was removed and 100 μl phospate-buffered saline (PBS) (PanEco, Russia) was added to the sediment and carefully resuspended by pipetting.

The resulting EVs were examined using the negative contrast method [20]. A 30-μl EVs suspension in PBS was applied to a 150 mesh copper grid coated with butvar film; after 1 min, excess liquid was removed by touching grid edge

with filter paper. Next, 30 μl of a 2% phosphotungstic acid neutralized with a 1 M NaOH to pH 6.5, was applied to the grid. Excess liquid was removed after 10 s in a similar manner. The grids were air-dried for 10 min and examined (an electron microscope JEM-100CXII, JEOL, Japan; instrumental magnifications ×27000, ×40000 and ×50000). The exosomes collected from 50 ml suspension of animal MSCs were assessed by the size (diameter) and counted at least in 10 fields of view per preparation in triplicate.

Samples of EVs from CM of canine MSCs (AT), mouse embryonic fibroblasts STO line, and bovine MSCs (BM) (negative control) were lysed (RIPA buffer, Wuhan Servicebio Technology, Ltd., China). The protein concentration in the lysates was measured using a protein assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Inc., USA). A 5× buffer (10% SDS, 250 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 50% glycerol, 5% β-mercaptoethanol) was added to equal volumes of lysates at 1:4 and heated for 10 min at 95 °C for reduction and denaturation. Proteins were sedeparated electrophoretiaacally in 10% polyacrylamide gel with 1% SDS (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Merck Millipore, USA). Membranes were blocked with 5% skim milk in PBS with 0.1% Tween-20 for 1 h at room temperature. Next, they were incubated at the same temperature for 1 h with primary antibodies (AB) to TSG101 (sc-7964, 1, Santa Cruz Biotenology, USA; dilution 1:1000) followed by 1 h incubation at room temperature with secondary goat AB to mouse antigens (AG) labeled with horseradish peroxidase (Axioma BIO, Russia).

Data statistical processing was carried out using Microsoft Excel.Arithmetic means (*M*) and standard errors of the means (±SEM) were calculated. The significance of differences was assessed using Student's *t*-test at $p < 0.05$.

Results. EVs were isolated from the CMs of MSCs of five animal species, for cattle and sheep the cell populations were MSCs (AT) and MSCs (BM). The cells had similar properties in vitro.

Figure 1 shows the fibroblast-like morphology of MSCs isolated from the BM of cattle (a) and sheep (g), from AT of cattle (d) and dogs (m), and UC of horses (k). Due to the lack of specific antibodies to AG of farm animals, the MSC phenotype was confirmed functionally by the ability to generate adipose and bone tissue cells upon induction. After induction by an adipogenic medium for 21 days, MSCs gradually transformed from fibroblast-like cells into flattened cells, and lipid droplets of different sizes appeared in the cytoplasm, which were stained red with fat red O (see Fig. 1, b, e, h, k, n). After 21-day incubation in an osteogenic medium, morphological changes appeared in all MSC populations, and silver-rhenium staining revealed phosphates and carbonates that turned black (see Fig. 1, c, f, i, l, o).

Each sample of isolated EVs was resuspended in 100 μl of PBS (assigned in triplicate). Electron microscopic analysis revealed round or irregularly shaped microparticles of different size and clusters of fibrous structures in all samples. The latter were probably dried substances contained in part of the EVs which were destroyed during preparation, since they were not fixed.

Analysis of the electron diffraction patterns showed that in all samples more than 70% of the preserved EVs had sizes from 50 to 100 nm, that is, they were classified as exosomes (Fig. 2). The diameters of the EVs did not differ statistically between different animal species ($p = 0.1$). When comparing the number of particles isolated from 50 ml CM of MSCs from different animal species, no statistically significant differences were found ($p = 0.1$). Thus, the EVs number per grid for bovine MSCs (BM) and MSCs (AT) were 3±0.1 and 6±0.07 of 50-100 nm particles, 7±0.02 and 4±0.03 of 100-150 nm particles, and 3±0.4 and

2±0.06 particles larger than 150 nm, respectively. EVs from CM of MSCs (AT) of dogs were the most homogeneous both in shape (round) and in size, the main part (12±0.02) were 50-100 nm particles. In samples isolated from CMs of sheep, cat, horse MSCs (AT) and horse MSCs (UC), the number of 50-100 nm particles was 7±0.2, 7±0.01; 5±0.7 and 8±0.02, respectively. From CM of sheep MSCs (BM) we isolated 2±0.1 particles up to 50 nm in size, 5±0.6 particles of 50-100 nm, 1±0.03 particles of 100-150 nm, 5±0.7 particles of 150-200 nm, 3±0.01 particles larger than 200 nm, two of which had an irregular shape, similar to ectosomes, budding microvesicles.

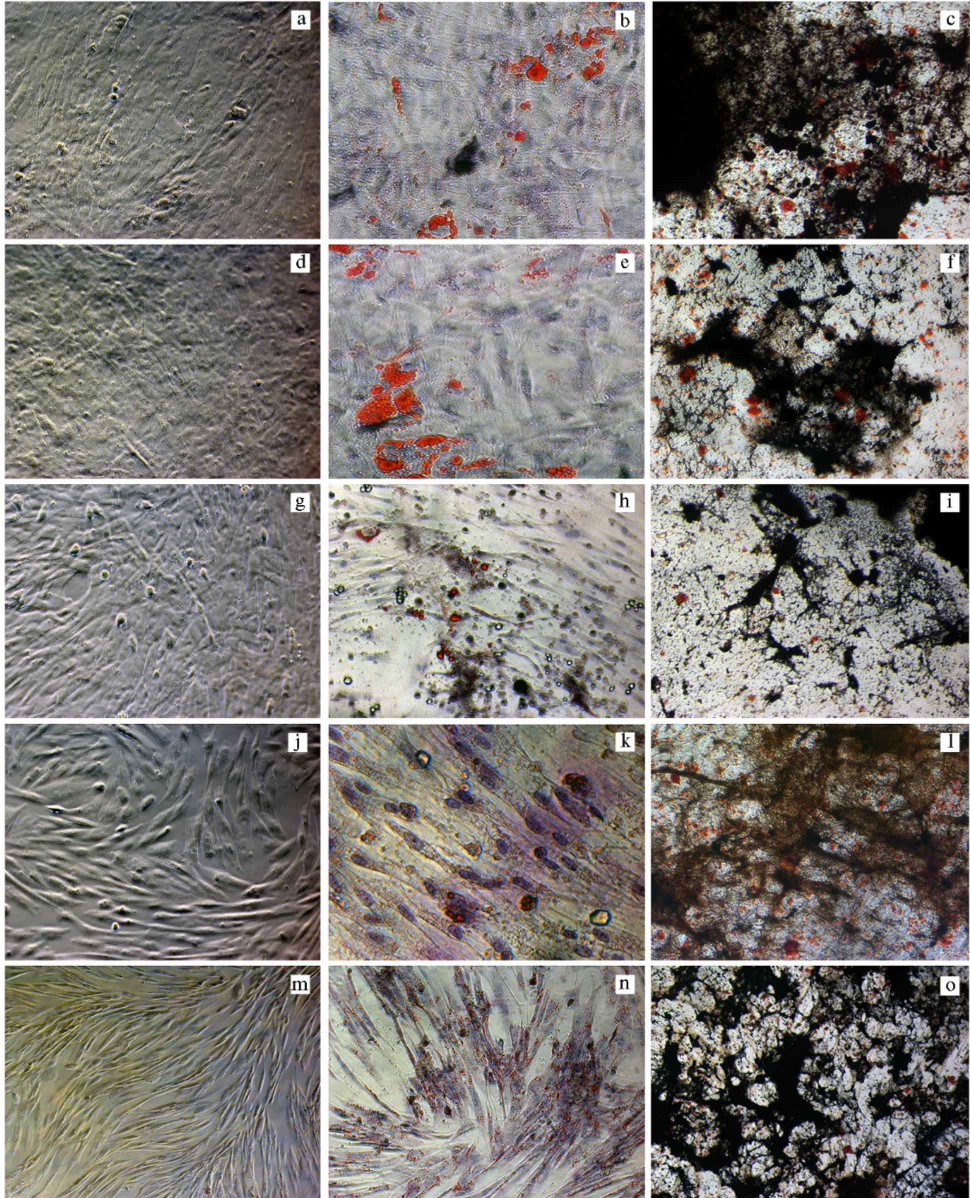

Fig. 1. Microphotographs of mesenchymal stem/stromal cells (MSCs) before collecting the conditioned medium for isolating exosomes (left, monolayer) **and their competence to adipo-** (fat red O staining, center) **and osteogenic** (silver-rhenium staining according to von Kossa, right) **differentiation upon induction:** a, b, c — MSCs from cattle bone marrow (BM), d, e, f — MSCs from cattle adipose tissue (AT), g, h, i — MSCs (BM) of sheep, j, k, l — MSCs from horse umbilical cord blood; m n, o — MSCs (AT) of a dog (microscope Carl Zeiss, Germany, magnification ×100; for b, e, j, k, ×200).

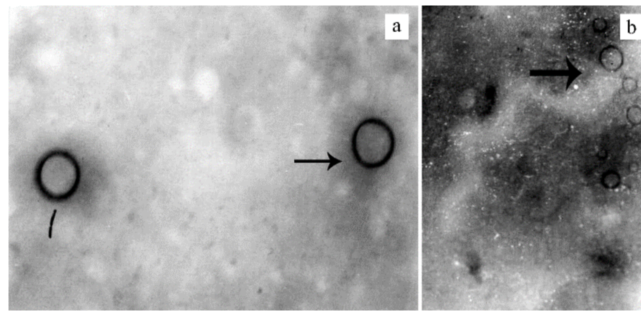

Fig. 2. Micrograph of extracellular vesicles from conditioned media of mesenchymal stem/stromal cells of cattle (a) **and sheep** (b) **bone marrow.** Arrows indicate exosomes (electronogram, JEM-100CXII microscope, JEOL, Japan, a — magnification ×54000, b — magnification ×50000).

To identify the phenotype of exosomes, EVs isolated from MSC CM of canine MSCs were used. This choice was due to the availability of mouse monoclonal antibodies to TSG101 recommended for the detection of TSG101 of mouse, rat, human and canine origin. Lysates of dog MSCs (AT) from which EVs were isolated, mouse embryonic fibroblasts STO line, and bovine MSCs (BM) (negative control) were used as controls. To identify exosomes, we used a protein marker for cellular exosomes and Western blot (immunoblot), an analytical method for determining specific proteins in a sample. Western blot is often used as a control test to confirm exosome isolation [7]. Surface proteins CD9, CD 63, CD81 can serve as markers of exosomes. We chose to detect the cytoplasmic protein TSG101 due to availability of the corresponding antibodies. As a result, in all samples, except for the negative control, 50-55 kDa bands stained with the anti-AG TSG101 antibody were found (Fig. 3).

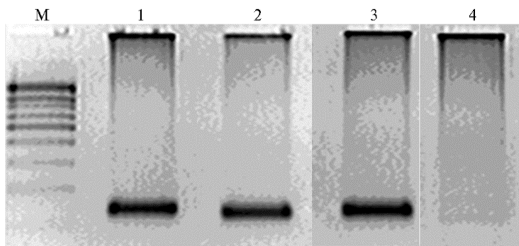

Fig. 3. TSG101 protein (Western blot) in samples isolated from canine adipose tissue (AT) **mesenchymal stem/stromal cell lysate** (1)**, extracellular vesicles from conditioned media of canine adipose tissue** (AT) **mesenchymal stromal cells** (2)**, mouse embryonic fibroblast STO lysate** (3)**, negative control** (4)**.** M — molecular weight marker, 8-195 kDa (Wuhan Servicebio Technology, Ltd., China).

In our work, we demonstrated that cells with the MSC phenotype from agricultural (cattle, sheep, horse) and small domestic (dog, cat) animals produce EVs which accumulate in the CM and can be extracted. Previously, exosomes were obtained from MSCs of horses [21], dogs [22], and cats [23]. Many methods are used to isolate EVs from the CV of MSCs [24-26] among which we can highlight differential centrifugation based on the difference in size and density between the EVs and other substances in the CM. This method is often used to extract exosomes due to the simplicity and stability of the result [27]. By DC method, unnecessary material is gradually removed from the CM. At the final stage, the purified CM is subjected to UC. UC is the most widely used method for exosome isolation and was once called the "gold standard" for exosome production [28].

We also used the DC method to obtain exosomes from animal MSC CM. In our study, we assessed the ultrastructure and particle size by electron microscopy. The results showed that EVs isolated from the CMs of MSCs (BM) and MSCs (AT) of all animal species are round or elliptical vesicles with membrane structures around the vesicles that are similar in shape. More than 70% of particles isolated from the CM of all MSCs had a size of 50-100 nm.

Exosomes can contain various proteins, the composition of which depends

on the host cell and is additionally modulated by its state (for example, stress or activation or inhibition of specific signaling pathways). Tetraspanins (CD9, CD63, and CD81) are the most common canonical exosome marker proteins present on the surface of vesicles. The surface localization of tetraspanin AG makes them suitable candidate targets for immunolabeling and purification of exosomes from biological samples. It should be noted that the difficulty in characterizing animal EVs compared to humans is due to the lack of specific EV related or non-related markers that can be used to characterize EVs across species. In addition, there are no available tissue- or cell-specific markers for isolating EV subpopulations and marker genes that could be used in the study of nucleic acids associated with EVs. Exosomes are enriched with Tsg101 and Alix proteins which are components of the endosomal sorting complex necessary for their transport. The cytoplasmic protein Tsg101 (Tumor Susceptibility Gene 101), involved in the formation of multivesicular exosome bodies and considered another important exosome marker, was identified as a marker of exosomes in the isolated samples [29]. Western blotting showed that all samples isolated from dog MSCs (AT) were positive for this protein.

Thus, conditioned media of mesenchymal stem/stromal cells from bone marrow, adipose tissue and umbilical cord blood of agricultural (cattle, sheep, horse) and small domestic (dog, cat) animals contain extracellular vesicles, including exosomes. Since the size and density of most EVs and other cellular components overlap to some extent, it can be assumed that the differential centrifugation method does not isolate only exosomes, but rather results in an enrichment of the sample in exosomes. In our work, we described a DC-based method for isolating micro-EVs containing exosomes from five animal species. This will provide the basis for the use of exosomes in disease diagnosis and treatment of farm and domestic animals in the future.

REFERENCES

1. Viktorova E.V., Savchenkova I.P. Multipotent mesenchymal stem cells in clinical veterinary practice. *IOP Conference Series: Earth and Environmental Science*, 2020, 548: 072072 (doi: 10.1088/1755-1315/548/7/072072).
2. Platonova S.A., Korovina D.G., Viktorova E.V., Savchenkova I.P. Equine tendinopathy therapy using mesenchymal stem cells. *KnE Life Sciences*, 2021, 6(3): 533-541 (doi: 10.18502/kls.v0i0.8987).
3. Hade M.D., Suire C.N., Suo Z. Mesenchymal stem cell-derived exosomes: applications in regenerative medicine. *Cells*, 2021, 10(8): 1959 (doi: 10.3390/cells10081959).
4. Gowen A., Shahjin F., Chand S., Odegaard K.E., Yelamanchili S.V. Mesenchymal stem cell-derived extracellular vesicles: challenges in clinical applications. *Frontiers in Cell and Developmental Biology*, 2020, 8: 149 (doi: 10.3389/fcell.2020.00149).
5. Mathivanan S., Ji H., Simpson R.J. Exosomes: extracellular organelles important in intercellular communication. *Journal of Proteomics*, 2010, 73(10): 1907-1920 (doi: 10.1016/j.jprot.2010.06.006).
6. Bazzan E., Tinè M., Casara A., Biondini D., Semenzato U., Cocconcelli E., Balestro E., Damin M., Radu C.M., Turato G., Baraldo S., Simioni P., Spagnolo P., Saetta M., Cosio M.G. Critical review of the evolution of extracellular vesicles' knowledge: from 1946 to today. *Int. J. Mol. Sci.*, 2021, 22(12): 6417 (doi: 10.3390/ijms22126417).
7. Doyle L.M., Zhuo W.M. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosomeiIsolation and analysis. *Cells,* 2019, 8(7): 727 (doi: 10.3390/cells8070727).
8. Tran P., Xiang D., Tran T., Yin W., Zhang Y., Kong L., Chen K., Sun M., Li Y., Hou Y., Zhu Y., Duan W. Exosomes and nanoengineering: a match made for precision therapeutics. *Adv. Mater.*, 2020, 32(18): e1904040 (doi: 10.1002/adma.201904040).
9. Fang Y., Zhang Y., Zhou J., Cao K. Adipose-derived mesenchymal stem cell exosomes: a novel pathway for tissues repair. *Cell and Tissue Banking*, 2019, 20(2): 153-161 (doi: 10.1007/s10561-019-09761-y).
10. Doyle L.M., Wang M.Z. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells*, 2019, 8(7): 727 (doi: 10.3390/cells8070727).
11. Chen J., Liu R., Huang T., Sun H., Jiang H. Adipose stem cells-released extracellular vesicles as a next-generation cargo delivery vehicles: a survey of minimal information implementation, mass production and functional modification. *Stem Cell Res. Ther.*, 2022, 13(1): 182 (doi:

10.1186/s13287-022-02849-5).
12. Moccia V., Sammarco A., Cavicchioli L., Castagnaro M., Bongiovanni L., Zappulli V. Extracellular vesicles in veterinary medicine. *Animals*, 2022, 12(19): 2716 (doi: 10.3390/ani12192716).
13. Nahand J.S., Mahjoubin-Tehran M., Moghoofei M., Pourhanifeh M.H., Mirzaei H.R., Asemi Z., Khatami A., Bokharaei-Salim F., Mirzaei H., Hamblin M.R. Exosomal miRNAs: novel players in viral infection. *Epigenomics*, 2020, 12(4): 353-370 (doi: 10.2217/epi-2019-0192).
14. Zhou C., Tan L., Sun Y., Qiu X., Lia Y., Song C., Liu W., Nair V., Ding C. Exosomes carry microRNAs into neighboring cells to promote diffusive infection of newcastle disease virus. *Viruses*, 2019, 11(6): 527 (doi: 10.3390/v11060527).
15. Mao L., Liang P., Li W., Zhang S., Liu M., Yang L., Li J., Li H., Hao F., Sun M., Zhang W., Wang L., Cai X., Luo X. Exosomes promote caprine parainfluenza virus type 3 infection by inhibiting autophagy. *Journal of General Virology*, 2020; 101: 717-734 (doi: 10.1099/jgv.0.001424).
16. Volkova I.M., Viktorova E.V., Savchenkova I.P., Gulyukin M.I. Characteristic of mesenchymal stem cells, isolated from bone marrow and fatty tissue of cattle. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2012, 47(2): 32-38 (in Russ.).
17. Korovina D.G., Yurov K.P., Volkova I.M., Alekseenkova S.V., Vasil'eva S.A., Savchenkova E.A., Savchenkova I.P. *Konevodstvo i konnyy sport*, 2015, 6: 31-33 (in Russ.).
18. Korovina D.G., Volkova I.M., Vasil'eva S.A., Gulyukin M.I., Savchenkova I.P. *Tsitologiya*, 2019, 61(1): 35-44 (doi: 10.1134/S0041377119010036) (in Russ.).
19. Savchenkova I.P., Vasilyeva S.A., Korovina D.G., Shabeikin A.A., Gulyukin A.M. Characterization of mesenchymal stem cells isolated from feline and canine adipose tissue. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(2): 395-403 (doi: 10.15389/agrobiology.2019.2.395eng).
20. Brenner S., Horne R.W. A negative staining method for high resolution electron microscopy of viruses. *Biochimica et Biophysica Acta*, 1959, 34: 103-110 (doi: 10.1016/0006-3002(59)90237-9).
21. Klymiuk M.C., Balz N., Elashry M.I., Heimann M., Wenisch S., Arnhold S. Exosomes isolation and identification from equine mesenchymal stem cells. *BMC Vet. Res.*, 2019, 5(1): 42 (doi: 10.1186/s12917-019-1789-9).
22. Aguilera-Rojas M., Badewien-Rentzsch B., Plendl J., Kohn B., Einspanier R. Exploration of serum- and cell culture-derived exosomes from dogs. *BMC Vet. Res.*, 2018, 14(1): 179 (doi: 10.1186/s12917-018-1509-x).
23. Sung S.-E., Seo M.-S., Kang K.-K., Choi J.-H., Lee S., Sung M., Kim K., Lee G.-W., Lim J.-H., Yang S.-Y., Yim S.-G., Kim S.-K., Park S., Kwon Y.S., Yun S. Mesenchymal stem cell exosomes derived from feline adipose tissue enhance the effects of anti-inflammation compared to fibroblasts-derived exosomes. *Vet. Sci.*, 2021, 8(9): 182 (doi: 10.3390/vetsci8090182).
24. Soares M.T., Catita J., Martins R.I., A.B. da e Silva O., Henriques A.G. Exosome isolation from distinct biofluids using precipitation and column-based approaches. *PLoS ONE*, 2018, 13(6): e0198820 (doi: 10.1371/journal.pone.0198820).
25. Huang L.-H., Rau C.-S., Wu S.-C., Wu Y.-C., Wu C.-J., Tsai C.-W., Lin C.-W., Lu T.-H., Hsieh C.- H. Identification and characterization of hADSC-derived exosome proteins from different isolation methods. *J. Cell. Mol. Med.*, 2021, 25(15): 7436-7450 (doi: 10.1111/jcmm.16775).
26. Helwa I., Cai J., Drewry M.D., Zimmerman A., Dinkins M.B., Khaled M.L., Seremwe M., Dismuke W.M., Bieberich E., Stamer W.D., Hamrick M.W., Liu Y. A comparative study of serum exosome isolation using differential ultracentrifugation and three commercial reagents. *PLoS ONE*, 2017, 12(1): e170628 (doi: 10.1371/journal.pone.0170628).
27. Kurian T.K., Banik S., Gopal D., Chakrabarti S., Mazumder N. Elucidating methods for isolation and quantification of exosomes: a review. *Molecular Biotechnology*, 2021, 63(4): 249-266 (doi: 10.1007/s12033-021-00300-3).
28. Coughlan C., Bruce K.D., Burgy O., Boyd T.D., Michel C.R., Garcia-Perez J.E., Adame V., Anton P., Bettcher B.M., Chial H.J., Königshoff M., Hsieh E.W.Y., Graner M., Potter H. Exosome isolation by ultracentrifugation and precipitation and techniques for downstream analyses. *Current Protocols in Cell Biology*, 2020, 88(1): e110 (doi: 10.1002/cpcb.110).
29. Koritzinsky E.H., Street J.M., Chari R.R., Glispie D.M., Bellomo T.R., Aponte A.M., Star R.A., Yuen P. Circadian variation in the release of small extracellular vesicles can be normalized by vesicle number or TSG101. *American Journal of Physiology-Renal Physiology*, 2019, 317(5): F1098-F1110 (doi: 10.1152/ajprenal.00568.2017).