*Ann. Anim. Sci., Vol. 25, No. 1 (2025) 189–200* *DOI: 10.2478/aoas-2024-0073*

sciendo

# EXTRACELLULAR VESICLES OBTAINED FROM EQUINE MESENCHYMAL STEM CELLS ISOLATED FROM ADIPOSE TISSUE IMPROVE SELECTED PARAMETERS OF STALLION SEMEN AFTER CRYOPRESERVATION*

Sebastian Sawicki[1♦], Artur Gurgul[2], Julia Gabryś[1], Barbara Kij-Mitka[1], Michał Bochenek[3], Elżbieta Karnas[4], Zenon Podstawski[1], Bogusława Długosz[1], Monika Bugno-Poniewierska[1]

[1]Department of Animal Reproduction, Anatomy and Genomics, University of Agriculture in Krakow, Mickiewicza 24/28, 30-059 Kraków, Poland
[2]Center for Experimental and Innovative Medicine, University of Agriculture in Krakow, Rędzina 1C, 30-248 Kraków, Poland
[3]Jagiellonian University, Malopolska Centre of Biotechnology, Gronostajowa 7A, 30-387 Kraków, Poland
[4]Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
♦Corresponding author: sebastian.sawicki@student.urk.edu.pl

**Abstract**

**Despite extensive research on the cryopreservation of stallion semen, the process still requires improvement. Several diluent additives have been developed to overcome cryopreservation issues, however, semen from valuable individuals often shows poor quality after thawing. Mesenchymal stem cells (MSCs) are regarded as factors that may play an important role in the regenerative process of sperm by secreting extracellular vesicles (EVs) that are actively involved in intercellular communication, tissue repair and cell membrane maintenance. The study aimed to determine the effect of the addition of EV-MSCs obtained from equine adipose tissue on various parameters of cryopreserved stallion semen. All cells met the minimum criteria established by the ISCT, being a characteristic surface profile of the presence of CD90 and lack of MHC II, differentiation into three mesodermal lineages, and adherence. EVs were collected from the expanded cells by ultracentrifugation, and their characteristics were confirmed using nanoparticle tracking analysis (NTA) and flow cytometry to detect CD63 and CD81 antigens. Semen was collected from 11 stallions of the Hucul breed, and each parameter of sperm quality with (25 µg/ml, 50 µg/ml) and without EV addition (motility, morphology and viability) was analyzed. The results showed statistically significant differences in sperm motility, progressive movement and viability following EV treatment. While the amplitude of lateral head displacement (ALH) and morphology parameters did not change, the addition of EVs does not affect these sperm features. The results provide an initial insight into the role of EVs in the maintenance and regeneration of horse sperm after cryopreservation-induced shock.**

**Key words: MSC, extracellular vesicles, differentiation, sperm parameters, cryopreservation**

The use of frozen semen in breeding practice as well as international trade is rapidly growing. Frozen semen guarantees the on-demand availability of semen and allows it to be transported over long distances. The cryopreservation process allows for the creation of sperm banks in order to preserve the genetic diversity of conservative breeds of primitive horses covered by the program of protection of genetic resources. Hucul horses are one of the Polish primitive breeds for which natural mating is used and artificial insemination is permitted with the approval of the studbook committee, therefore little is known about the physiological parameters of semen of this breed or its stability in the cryopreservation process.

Studies of Hutsul stallions semen, collected in the semen bank of the University of Agriculture in Krakow, showed a significant decrease in semen parameters after cryopreservation, proving the sensitivity of semen of this breed to the cryopreservation process (Bugno-Poniewierska et al., 2021) therefore, ways to improve semen quality should be sought after thawing.

The cryopreservation process may cause deleterious changes in sperm morphology resulting from thermal, mechanical, chemical, osmotic, and oxidative damage (Alvarez and Storey, 1992; Lançoni et al., 2018). Generally, only approximately 50% of sperm survive this process (de Andrade et al., 2018). Factors that contribute to sperm damage during cryopreservation include temperature fluctuations throughout cooling and freezing processes, formation of ice crystals, changes in the plasma

*This study was financed from the University of Agriculture in Krakow, grant number SUB020013-D015.

membrane, and production of reactive oxygen species (ROS) (Amann and Pickett, 1987). Cryopreserved sperm also undergo accelerated and premature aging (Contreras et al., 2020).

The impact of the cryopreservation process on the reduction of stallion semen quality prompted research aimed at improving this process through the addition of various substances that act as cryoprotectants to diluents, e.g., egg yolk (Pillet et al., 2011), soy lecithin (Papa et al., 2011), liposomes (Medina-León et al., 2019), glycerol (Alvarenga et al., 2005), amides (Wu et al., 2015), non-enzymatic antioxidants (mainly GSH) (Oliveira et al., 2013), manganese (III) porphyrin tetrakis (benzoic acid 4–69) (MnTBAP) (Treulen et al., 2019) and resveratrol (Nouri et al., 2018).

Extenders protect sperm and allow them to maintain motility and fertility by stabilizing the plasmalemma, providing energy substrates and preventing the deleterious effects of pH and osmolarity changes (Foote and Leonard, 1964). Such additives contribute to improving the quality of semen after cryopreservation and increasing the rate of sperm fertilization (Baiee et al., 2018).

A widely held assumption is that the achievements of regenerative medicine can also be used to protect sperm from the adverse effects of environmental factors and regenerate them after cryopreservation. One of the modern regenerative medicine tools is mesenchymal stem cells (MSC), which, due to their ability to secrete paracrine growth factors, cytokines, antifibroscopic factors and angiogenic mediators, have the potential to regulate cell and tissue repair systems (Meirelles et al., 2009). MSCs, also known as mesenchymal stromal cells, are spindle-shaped, self-renewing cells with multipotent abilities (they can differentiate into chondrocytes, osteocytes and adipocytes). The minimum criteria for defining human MSCs established by the International Society for Cellular Therapy (ISCT) require that mesenchymal stem cells demonstrate the ability to differentiate into adipocytes, osteocytes, and chondrocytes, confirmation of specific surface antigens, and adhesion to plastic (Dominici et al., 2006). These cells are derived from fetal tissues (umbilical cord, umbilical cord blood, and placenta) and various postnatal tissues such as adipose tissue or bone marrow, which are the main sources of these cells, they adhere to tissue culture plates and have certain surface markers that enable their identity (Jiang and Xu, 2020). The ability to self-renew (Taylor et al., 2007), and the ability to undergo multidirectional differentiation (Chen et al., 2008) and modulate the immune system (Barberini et al., 2014) made MSCs widely used in regenerative medicine.

The MSC secretome consists of a free fraction, including soluble factors and metabolites, and a fraction enclosed in extracellular vesicles (EV). These EVs contain various bioactive molecules (mRNAs, miRNAs, proteins, and lipids) and function as transmitters in long-distance intercellular communication (Pavani et al., 2018) while regulating various physiological processes such as cell proliferation, differentiation, and migration (Zhang et al., 2016). EVs, including exosomes, ectosomes, and apoptotic vesicles, have been used as therapeutic delivery systems (Gangadaran et al., 2017 a).

Due to their biogenesis, the composition of the EV membrane most closely resembles the plasma membrane of stem cells (Yáñez-Mó et al., 2015). These vesicles are a heterogeneous group of particles secreted by all cell types that, according to the latest findings of the minimum information for extracellular vesicle research updated in 2018 (MISEV2018), can be classified according to their size ("small EV" <100 nm or <200 nm, "medium/high EV" >200 nm), biochemical composition or origin (Théry et al., 2018).

Research suggests that EVs play an important role in the regulation of physiological processes such as stem cell maintenance, tissue repair, and immune modulation (Basu and Ludlow, 2016). They can be found in body fluids such as blood, urine, and plasma. Furthermore, EVs are also secreted into the cell culture medium (Gangadaran et al., 2017 b). In addition, EVs have been shown to exert paracrine effects during stem cell therapy (Davis, 2016), and MSC-derived EVs (MSC-EV) may be essential for the therapeutic effects of MSCs (Ophelders et al., 2016; Nakano et al., 2016).

The contents of the exosomes are transported not only to the surrounding cells but also to more distant tissues. After the uptake of a specific cargo (i.e., proteins, lipids, mRNA, miRNA) from an EV, the function or phenotype of the target cell, and even cell homeostasis, is modified through the regulation of cellular signaling, enzymatic reaction pathways, or other cellular mechanisms (Grzybowska et al., 2019).

All these features of MSCs and EVs prompted the hypothesis that the addition of EVs would serve as a protective factor that can improve sperm protection during cryopreservation and ameliorate semen parameters after thawing.

To our best knowledge, there is no published data on the effect of MSC-derived EVs on the improvement of stallion semen after cryopreservation. The research hypothesis assumes that the addition of EVs obtained from MSCs will improve the stallion semen quality after cryopreservation.

Therefore, the study aimed to determine whether the addition of EVs derived from equine MSC isolated from adipose tissue improved the parameters of cryopreserved stallion semen.

## Material and methods

### Study design

Mesenchymal stem cells isolated from adipose tissue were identified by showing the adherence ability, performing trilineage differentiation and by using a flow cytometer to confirm the presence of a characteristic positive surface marker – CD90 and the lack of a negative surface marker – MHCII.

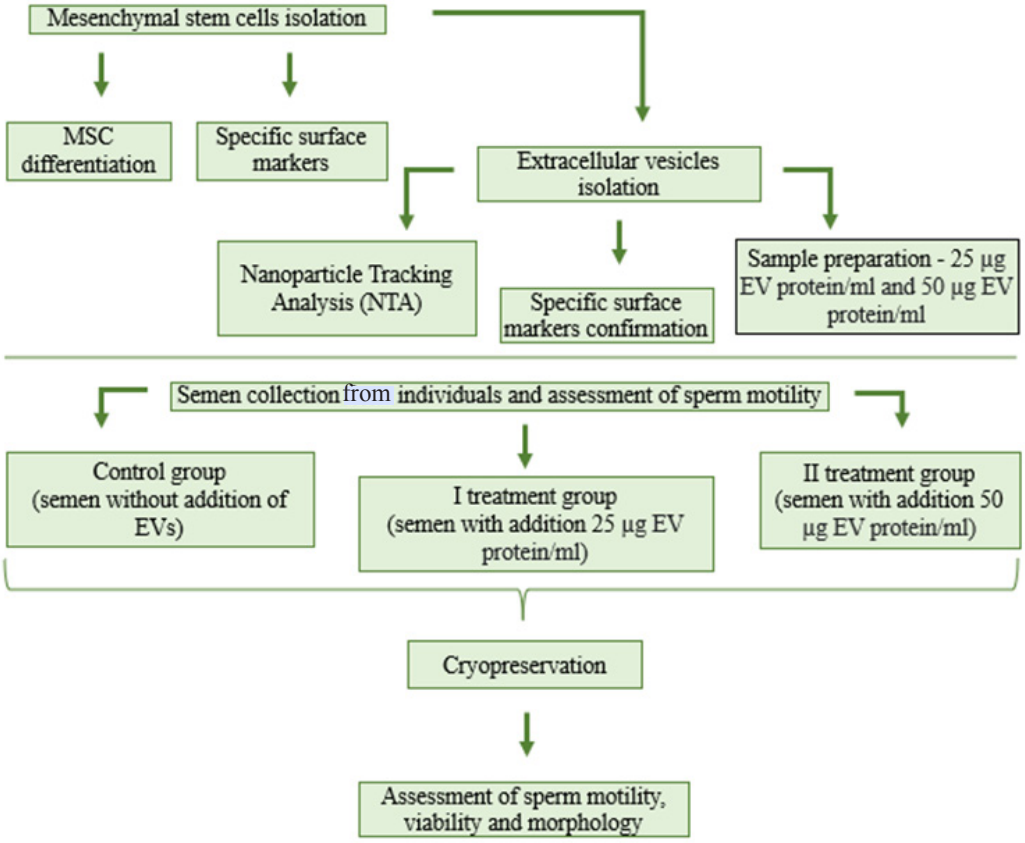



Figure 1. Diagram of the experiment steps

From the confirmed MSCs culture media, extracellular vesicles were isolated by ultracentrifugation. The EVs identification was carried out using NTA analysis to determine the size and concentration of EVs, and using a flow cytometer to confirm positive surface markers – CD63 and CD81. The obtained extracellular vesicles were divided into two groups – 25 µg EV protein/ml and 50 µg EV protein/ml.

Semen was collected from 11 Hucul stallions. General sperm motility was checked before freezing using the SCA system. Semen from each individual was divided into three groups (control group and two treatment groups). The control group was sperm sample without the addition of extracellular vesicles, while the first treatment group was sperm sample with the addition of 25 µg EV protein/ml, and the second one was sperm sample with the addition of 50 µg EV protein/ml. The semen was frozen, and after the cryopreservation process, the motility, viability and morphology of sperm from each group for each individual were checked using SCA system. A diagram showing the steps of the experiment is shown in Figure 1.

## Material and methods

The material for this study was adipose tissue collected from mare of unregistered age and breed. The sample was collected post-mortem at the slaughterhouse as part of commercial horse slaughter in accordance with EU regulations, therefore, the approval of the local ethics committee was not required for this procedure. EVs were obtained from MSCs at a rate of 25 µg EV protein/ml and 50 µg EV protein/ml.

The semen for this study came from Hucul stallions from the stud in Gładyszów in Poland, which were bred under equal conditions, the same environmental conditions, maintenance and nutrition of the animals.

Single ejaculates from 11 stallions were collected into an artificial open vagina, which allowed separation of the sperm-rich fraction. Semen was collected in February on the same day from all individuals. The collected ejaculates were diluted 1:1 using EquiPlus extender (Insatex-MT, Poznań, Poland), placed in sterile falcons and transported at 4°C to the laboratory. Semen was diluted 1:5 with EquiPlus (Minitube, Tiefenbach, Germany) and assessed immediately after collection for volume, color, and general sperm movement. The addition of extracellular vesicles and the freezing procedure were performed within 24 hours of material collection.

### Adipose tissue harvesting

The horse adipose tissue was obtained post-mortem from the periuterine area. Approximately 10 g of tissue was excised, avoiding blood vessels, and transferred to

a 50 ml Falcon tube with 30 ml of sterile Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Darmstadt, Germany) containing 2% antibiotic. Immediately after collection, the sample was transported to the laboratory at 4°C and processed within 4 hours of collection.

**MSC culture from adipose tissue**

The collection and culture of MSCs from adipose tissue was carried out using a previously validated method (Bundgaard et al., 2018). After several washes with sterile PBS, the adipose tissue was digested in enzyme medium (DMEM 1 g/L glucose, L-glutamine, sodium bicarbonate, and phenol red) (Sigma-Aldrich, Darmstadt, Germany), supplemented with 1% penicillin/streptomycin solution (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany) with 1.5 mg/ml collagenase type I (Thermo Fischer Scientific) added to the media, which was filtered through a 0.2 µm filter (Corning). The adipose tissue was digested for 3 hours at 37°C in an orbital shaker at 30 rpm.

The medium with the released cells was filtered through a cell filter (70 µm) (Corning) to remove undigested adipose tissue residues and then centrifuged at 400 × *g* for 10 min. The supernatant was discarded, and the cell pellet was washed with sterile PBS. The cell pellet was resuspended in sterile culture medium (DMEM 1000 mg/L glucose, L-glutamine, and sodium bicarbonate, phenol red) (Sigma-Aldrich, Darmstadt, Germany) supplemented with 1% penicillin/streptomycin solution (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany) and distributed in four culture dishes and grown at 37°C in a humidified atmosphere containing 5% $CO_2$.

After 48 h, non-adherent cells were removed, and fresh medium was added. The medium was changed every 2 to 3 days, and cells were passaged (P1) at 70 to 80% confluence (7 to 10 days) using trypsin-EDTA (0.25%) (Thermo Fisher Scientific). Cells were seeded in a ratio of 1:3. After the first passage, cells were further expanded in DMEM (Sigma-Aldrich, Darmstadt, Germany) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany).

At 70 to 80% confluence, cells were trypsinized with trypsin-EDTA (0.25%) (Thermo Fisher Scientific) and frozen in FBS (Sigma-Aldrich, Darmstadt, Germany) with 10% dimethyl sulfoxide (Sigma-Aldrich, Darmstadt, Germany) in aliquots of 0.5 million cells/ml at -80°C and then in liquid nitrogen for preservation and long-term storage.

Cryopreserved cells were thawed and propagated in DMEM supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany). At 70 to 80% confluence, cells were re-passaged with trypsin-EDTA (0.25%) (Thermo Fisher Scientific) and plated at a ratio of 1:3. At passage 4, cells at 70% confluence were used for differentiation into mesodermal lineages (chondrogenic, osteogenic and adipogenic) and to check for the presence of stem cell-specific surface markers using flow cytometry.

**MSC differentiation**

Adipogenic, osteogenic and chondrogenic differentiation was carried out in a monolayer. Cells were passaged (P3) in 24-well plates at a density of approximately $2\times10^3$ cells/well and expanded in DMEM supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany) and grown at 37°C in a humidified atmosphere containing 5% $CO_2$. Medium was changed every 2 to 3 days, and after reaching ~70% confluence, cells were washed with sterile PBS and the medium was changed to adipogenic differentiation medium (DMEM 1 g/L glucose, L-glutamine and sodium bicarbonate, without phenol red) (Sigma-Aldrich, Darmstadt, Germany) supplemented with 2% FBS (Sigma-Aldrich, Darmstadt, Germany), 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany), $10^{-7}$ M dexamethasone (Sigma-Aldrich, Darmstadt, Germany), 1:100 v/v insulin-transferrin-selenium (ITS) (Thermo Fisher Scientific), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Darmstadt, Germany) and 100 µM indomethacin (Sigma-Aldrich, Darmstadt, Germany), osteogenic media (DMEM 1 g/l glucose, L-glutamine and sodium bicarbonate, no phenol red) (Sigma-Aldrich, Darmstadt, Germany) supplemented with 2% FBS (Sigma-Aldrich, Darmstadt, Germany), 1% (v /v) ITS (Thermo Fisher Scientific), 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany), $10^{-7}$ M dexamethasone (Sigma-Aldrich, Darmstadt, Germany), 50 µg/mL ascorbic acid (L-ascorbic acid 2-phosphate) (Sigma-Aldrich, Darmstadt, Germany), 10 µl/ml of 1 M ß-glycerophosphate (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany) or chondrogenic differentiation medium (DMEM 4.5 g/l glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, with phenol red) (Sigma-Aldrich, Darmstadt, Germany) supplemented with 2% FBS (Sigma-Aldrich, Darmstadt, Germany), 1% (v/v) ITS (Thermo Fisher Scientific), 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany), $10^{-7}$ M dexamethasone (Sigma-Aldrich, Darmstadt, Germany), 50 µg/ml ascorbic acid (Sigma-Aldrich, Darmstadt, Germany), 10 ng/ml TGF-β3 (recombinant human TGF-β3) (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany). The differentiation medium was changed every 3 days for 21 days. Confirmation of adipogenic differentiation was performed by staining with Oil Red O (Sigma-Aldrich, Darmstadt, Germany), chondrogenic with 0.1% Safranin O (Sigma-Aldrich, Darmstadt, Germany) and osteogenic with 2% Alizarin Red, pH 4.2 (Sigma-Aldrich, Darmstadt, Germany).

**Flow cytometry for MSCs**

Cells (P4) were grown to approximately 80% confluence and harvested for flow cytometry analysis. Before

staining for surface antigens, cells were incubated in 10% FBS (Sigma-Aldrich, Darmstadt, Germany) in PBS (Sigma-Aldrich, Darmstadt, Germany) for 10 min to reduce non-specific binding and then washed with PBS (Sigma-Aldrich, Darmstadt, Germany). All surface antigens were then stained for 30 minutes using primary antibodies against CD90 (5E10; Thermo Fisher Scientific #A15726) and MHC II (CVS20; Thermo Fisher Scientific #MA5-28491), both antibodies were commercially conjugated. After staining and two more washes in PBS, the cells were resuspended in Dulbecco's phosphate buffered saline (dPBS) (Sigma-Aldrich, Darmstadt, Germany) and stored at 4°C until analysis.

For all samples, at least 10,000 cells were analyzed using a flow cytometer (Beckman Coulter Navios; Software Kaluza 2.1); lasers: 488 nm for FITC, 638 nm for APC; optical filters: 550DCSP + 525BP for FITC, 710DCSP + 660BP for APC; flow rate set to "MEDIUM"; calibration QC – SPHERO Ultra Rainbow (Spherotech) beads before each session. All data were corrected for autofluorescence as well as non-specific binding using isotype-matched negative controls. All isotypes were matched to an immunoglobulin (Ig) subtype, conjugated to the same fluorochrome, and used at the same fluorescence/protein concentration as the corresponding Ab. Fluorescence was presented as PEAK signals on logarithmic scale. No compensation was necessary between FITC and APC signals.

**Isolation of extracellular vesicles**

The isolation of EVs was performed based on previously validated methods (Klymiuk et al., 2019; Gabryś et al., 2022). Equine AT-MSCs (P4) were proliferated in DMEM (Sigma-Aldrich, Darmstadt, Germany) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany) and 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany) and grown at 37°C in a humidified atmosphere containing 5% $CO_2$ to approximately 70 to 80% confluence. Then cells were washed with sterile dPBS (Sigma-Aldrich, Darmstadt, Germany) and cultured in exosome-depleted medium (DMEM 1 g/L glucose, L-glutamine, and sodium bicarbonate, phenol red) (Sigma-Aldrich, Darmstadt, Germany), supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany), 0.1% amphotericin (Sigma-Aldrich, Darmstadt, Germany), 2% exosome-depleted FBS (Thermo Fisher Scientific) and 1× ITS (Thermo Fisher Scientific) for 72 h. The supernatant was collected and subjected to various centrifugation steps at 4°C starting at 2000 × *g* for 10 min, then at 10000 × *g* for 30 min to remove dead cells and protein aggregates.

The supernatant was collected without disturbing the pellet and filtered through a 0.2 µm filter (Corning). The supernatant was then further ultracentrifuged using a fixed-angle rotor (70 Ti-Beckman) at 120,000 × *g* for 70 min at 4°C. After pooling the samples and supplementing them with dPBS (Sigma-Aldrich, Darmstadt, Germany), the samples were again ultracentrifuged at 120,000 × *g* for 70 min at 4°C. After ultracentrifugation, the EVs were left in dPBS (Sigma-Aldrich, Darmstadt, Germany) and frozen at –80°C for later use. To obtain 25 and 50 µg/ml of EV proteins, samples were measured by quantifying the protein content using the Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

**Nanoparticle tracking analysis**

Nanoparticle tracking analysis (NTA) with NanoSight NS500 (Malvern Instruments Ltd.) was performed to determine the concentration and size of EVs in the cell medium after ultracentrifugation. Samples of the EVs obtained previously were diluted in dPBS (Sigma-Aldrich, Darmstadt, Germany) prior to analysis to achieve their optimal concentration. A digital camera (sCMOS) collected laser light (405 nm) scattered by particles in the tested suspension through the lens of the microscope. EV motion analysis software made it possible to simultaneously analyze many individual particles and calculate their hydrodynamic averages based on the Stokes-Einstein equation. Data were analyzed using NTA 3.2 Dev Build 3.2.16 (Malvern) analysis software and presented as the mean ± standard deviation of triplicate experiments.

**Flow cytometry for EVs**

Detection of EV surface markers was performed by high-resolution Apogee A60-Micro-PLUS flow cytometer and Histogram software (Apogee Flow Systems, Hemel Hempstead, UK). EV samples were stained with APC-conjugated antibodies against CD63 (clone MEM-259; Thermo Fisher Scientific) and CD81 (clone 5A6; BioLegend, CA, USA), or the appropriate isotype control (IgG1k APC mouse, Miltenyi Biotec, Bergisch Gladbach, Germany). The antibodies (5 µl per test) were suspended in 0.2 µm-filtered dPBS without $Ca^{2+}$ or $Mg^{2+}$ (Lonza, Basel, Switzerland) and centrifuged at 21,000 × *g* for 20 min at 4°C to remove potential debris and protein aggregates. Obtained supernatants of staining buffers were transferred to the fresh tubes and $1 \times 10^8$ of EVs were added per test. Samples were then stained for 20 min at 4°C and subjected to the acquisition.

**Semen freezing**

The semen intended for freezing was centrifuged from the EquiPlus extender (Insatex-MT, Poznań, Poland) and then diluted with the SBS Cryosystem Spectrum Red extender (Insatex-MT, Poznań, Poland). EVs from MSCs were added to two treatment groups (equivalent to 25 and 50 µg EV protein/ml). Sperm samples without and with extracellular vesicles were filled into straws using semi-automatic filling and sealing machine SFS133 (Minitube, Tiefenbach, Germany).

Aliquots of semen (0.25 ml) were cooled to 5°C/30 min, and then the straws were placed in liquid nitrogen vapor for 15 min. Then, the semen straws were stored in liquid nitrogen until analysis.

**Semen preparation for analysis using the SCA system**

Semen from two pooled straws was used for analysis. The straws were heated at 37°C for 30 seconds. Semen was used to analyze sperm motility and viability. Sample was centrifuged at 2000 rpm for 7.5 min, the supernatant was discarded, and 1 ml of warm PBS (Sigma-Aldrich, Darmstadt, Germany) (37°C) was added to remove extender and allow the dye to penetrate the sperm. Then sperm morphology was analyzed by microscope.

**Sperm motility analysis**

The suspensions prepared in the previous section were applied to Goldcyto microscope slides (TK Biotech, Warsaw, Poland). Sperm motility was determined in the Sperm Class Analyzer (SCA) system using the "Motility" module at 10× magnification using phase contrast using microscope Nikon Eclipse E200. The general and progressive movement of spermatozoa was assessed, as well as the following parameters, namely velocity curvilinear (VCL μm/s), velocity straight line (VSL μm/s), velocity average path (VAP μm/s), linearity (LIN %), straightness (STR %), wobble (WOB %), and amplitude of lateral head displacement (ALH μm). Depending on the sperm concentration, 3–5 images were taken to capture a minimum of 500 sperm.

**Sperm viability analysis**

The FluoVit kit (TK Biotech, Warsaw, Poland) was used to analyze sperm viability. One μl of BLUE (trihydrochloride trihydrate) preheated at 37°C was added to 10 μl of the semen sample and incubated at 37°C for 5 minutes. Then 1 μl of RED (propidium iodide), also pre-warmed at 37°C was added and incubated at room temperature for 5 minutes. The stained sample (10 μl) was placed on a standard slide, covered with a coverslip, and analyzed by fluorescence microscopy. The analysis was performed using the "Vitality" module in the SCA system using microscope Nikon Eclipse E200. In each of the tested samples, minimum 300 randomly selected spermatozoa were evaluated.

**Sperm morphology analysis**

Sperm sample (10 μl) was collected, placed on a glass slide, and a smear was made. The dried slides were stained with Rapid Sperm Blue (TK Biotech, Warsaw, Poland) according to the manufacturer's instructions. Sperm morphology was assessed in the SCA system using the "Morphology" module. The analysis was performed at 60× magnification using white light with a blue filter using microscope Nikon Eclipse E200. In each of the tested samples, minimum 100 randomly selected spermatozoa were evaluated. Morphological parameters were determined automatically and concerned the head, acrosome, insert and tail of the sperm.

**Statistical analysis**

Statistical data analysis was performed using the free JASP program. The normality of the distributions was tested using the Shapiro-Wilk test. Friedman's test was used to assess the statistical significance of differences between the groups with and without the addition of EVs. A p-value of $<0.05$ was considered statistically significant. If the p-value was significant, an appropriate post hoc multiple comparison test (Bonferroni test) was performed using the Holm-Bonferroni correction. All results are presented as means or percentages unless otherwise noted.

## Results

**MSC isolation and culture**

Primary cell cultures were obtained from collected adipose tissue samples following digestion with collagenase. Adhesion to the culture plastic occurred approximately 48 to 72 hours after seeding, and the isolated cells showed a fibroblast-like shape with a central nucleus and abundant cytoplasm. Subsequent passages were made after cultures reached 70 to 80% confluence each time. Fibroblast-like cells were observed in all passages.

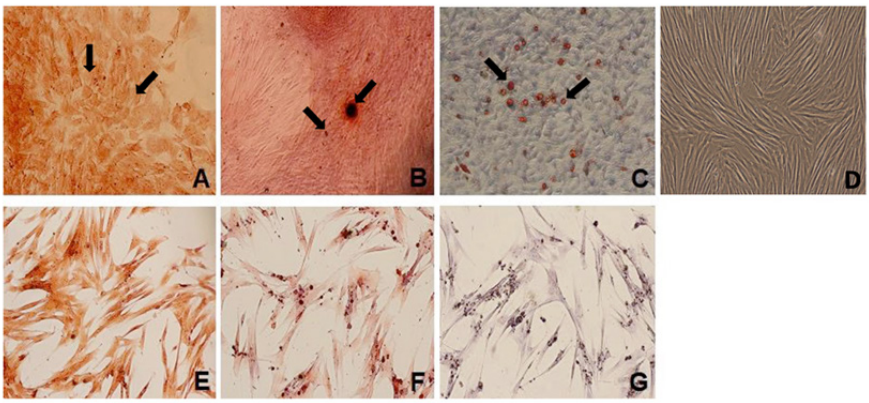



Figure 2. Results of chondrogenic, osteogenic, adipogenic differentiation of MSCs after 3 passages (A) Safranin O staining after 21 days in chondrogenic differentiation medium (B) Alizarin Red cell staining after 21 days in osteogenic differentiation medium (C) Oil Red staining O, Hematoxylin after 14 days of culture in adipogenic differentiation medium (D) MSCs after 3 passages (E) Safranin O staining in a control sample (F) Alizarin Red staining in a control (G) Oil Red O staining, Hematoxylin-stained control; (arrows in the figures sequentially indicate stained (A) intracellular proteoglycans (B) calcified extracellular matrix deposits (C) lipid droplets

**MSC differentiation**

Trilinear differentiation has been documented for AT-MSCs after appropriate induction. Under the influence of the differentiation media, the fibroblast-like cells showed a tendency to change morphology from day 14 following induction. AT-MSCs successfully differentiated into adipocyte, osteocyte and chondrocyte lineages. Negative (non-induced) control cells showed no changes in morphology for each type of differentiation (Figure 2). On day 21 following induction, the use of Oil Red O staining (Sigma-Aldrich, Darmstadt, Germany) for lipids, Safranin O (Sigma-Aldrich, Darmstadt, Germany) for the staining of intracellular proteoglycans, and Alizarin Red (Sigma-Aldrich, Darmstadt, Germany) allowed the visualization of calcified deposits of the extracellular matrix (all structures marked with arrows in the pictures). For adipogenic differentiation, hematoxylin stained the cytoplasm and nucleus blue to identify lipid droplets in differentiated cells.

**Surface markers of AT-MSC**

Based on the minimum criteria for defining human MSCs (Dominici et al., 2006) and adipose-tissue derived stromal/stem cells (Bourin et al., 2013) established by the International Society for Cellular Therapy (ISCT), two of several characterized horse MSC-specific markers were selected: CD90, a positive marker for AT-MSC and MHC II, a negative marker for AT-MSC. Flow cytometry analysis showed the presence of cells with only one of the two markers on their surface, CD90 stained in 92.52% of the cells tested, which was in line with the expected results, while the number of cells with both markers was only 7.36%. The results of the flow cytometer analysis are shown in Figure 3.

**EV isolation and characteristics**

Using a Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) allowed the estimation of the final concentration of EVs, which was approximately 2 mg protein/ml. Subsequently, EVs were characterized in two ways. Firstly, Nanoparticular Tracking Analysis of EV-MSCs obtained by ultracentrifugation of the culture fluid showed that the size of extracellular vesicles obtained by this method ranged from 70 to 340 nm (Figure 4) with average size and standard deviation of 144.9±4.4, while the concentration of these vesicles was $6.74 \times 10^{11} \pm 9.99 \times 10^{10}$ particles/ml (Table 1). Secondly, high-resolution flow cytometry allowed the phenotypic characterization of EVs, with 10.6% and 22.4% of the resulting particles being positive for CD63 and CD81, respectively (Figure 5).

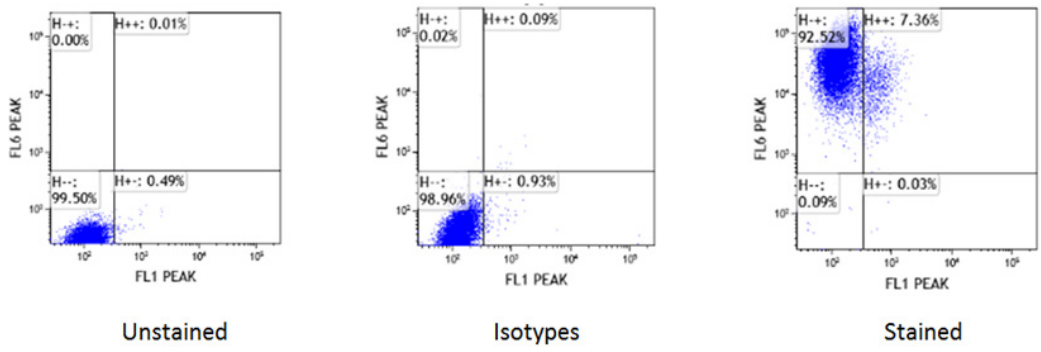



Figure 3. Analysis of mesenchymal stem cell surface markers by flow cytometry. The figure shows the results for control unstained cells, control cells stained with FITC and APC isotypes, and surface antigen MHC II (FL1 PEAK – FITC) and CD90 (FL6 PEAK – APC)

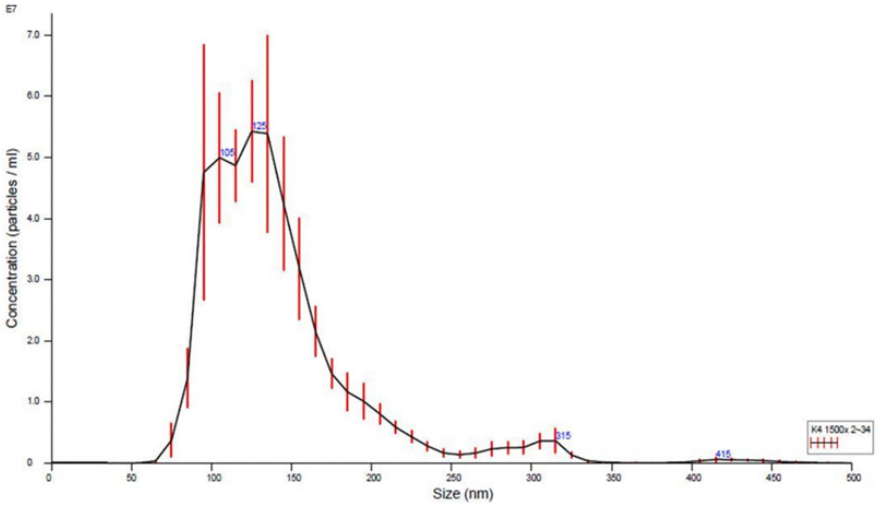



Figure 4. Nanoparticle tracking analysis (NTA). Representative analysis of EVs by NanoSight NS500 showing the concentration of EV-MSCs in particles per mL and the diameter of the EV-MSCs in nanometers (nm). The sample was analyzed in triplicate and red lines are standard error bars. Peaks are indicated by blue numbers (EVs dilution 1:1499)

Table 1. Mean size and concentration of extracellular vesicles with standard deviation in NTA analysis

| Mean ± stand. error (nm) | Mode ± stand. error (nm) | Concentration (particles/ml) |
|---|---|---|
| 144.9±4.4 | 112.3±5.2 | $6.74 \times 10^{11} \pm 9.99 \times 10^{10}$ |

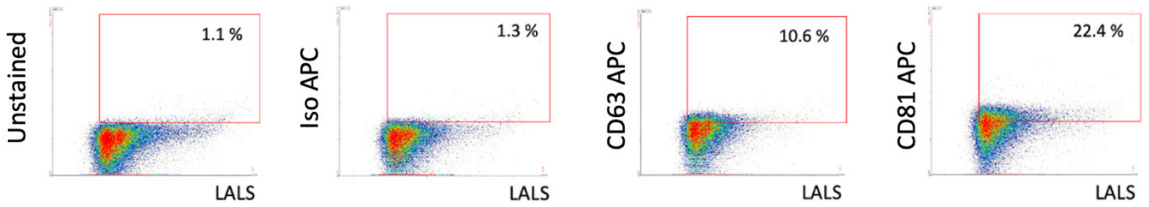



Figure 5. High-resolution flow cytometry of EV samples. Representative dot plots of EV-MSCs, collected with the A60-Micro-PLUS cytometer. The percentage of positive objects for the analyzed markers is shown in red gates. LALS – large-angle light scatter parameter, corresponding to the relative size of the analyzed particles

Table 2. Results of sperm motility before and after cryopreservation process for the control group

| Individual | Motility (%) | |
|---|---|---|
| | chilled semen | thawed semen |
| I | 97.63 | 9.44 |
| II | 98.81 | 33.23 |
| III | 61.67 | 13.42 |
| IV | 95.05 | 6.74 |
| V | 94.20 | 8.4 |
| VI | 46.45 | 31.85 |
| VII | 98.71 | 14.00 |
| VIII | 99.36 | 18.1 |
| IX | 36.35 | 37.62 |
| X | 33.29 | 20.39 |
| XI | 99.57 | 16.67 |

**Assessment of sperm motility before cryopreservation**

Sperm motility was checked in each semen sample collected from each individual using the SCA system. Sperm motility before the cryopreservation process was over 90% in most individuals (78.28±27.78% on average), while in some individuals sperm motility was clearly lowered in a range of 9–40%. The results of semen motility before and after freezing for the control group are presented in Table 2. For all samples, semen motility significantly decreased after cryopreservation to the mean level of 19.08±10.64%.

**Assessment of semen parameters after EVs addition**

After thawing the semen, sperm motility, viability and morphology were analyzed in each of the experimental groups. In all parameters of sperm motility, apart from ALH, an increasing trend was observed, i.e., the parameters are higher for semen with the addition of EV protein compared to the controls. However, statistically significant differences were observed only in overall sperm motility, progressive motility, and sperm viability (Table 3). In the case of sperm motility, the addition of EV proteins at a concentration of 25 µg/ml (23.125%) ($P<0.05$) and 50 µg/ml (24.668%) ($P<0.01$) resulted in statistically significant differences compared to the control (19.078%). The percentage of spermatozoa in progressive movement differed significantly between spermatozoa supplemented with EV proteins (25 µg/ml) (8.348%) and the control (6.650%) ($P<0.05$). Furthermore, the addition of 50 µg/ml EV proteins caused statistically significant differences in the percentage of spermatozoa with progressive movement (10.579%) in relation to the 25 µg/ml group and the control (6.650%) ($P<0.01$). For sperm viability, statistically significant differences were found for all groups at $P<0.05$ and between the control (60.185%) and sperm with the addition of EV proteins in the amount of 50 µg/ml (78.139%) ($P<0.01$). In the case of sperm morphology, the parameters did not show statistically significant differences between the study groups (Table 4).

Table 3. Changes in sperm motility and percentage of viable sperm assessed by SCA after adding EV protein (mean ± SD)

| | | EV protein dose | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 µg/ml | | 25 µg/ml | | 50 µg/ml | |
| Sperm parameter | n | mean | SD | mean | SD | mean | SD |
| Motile (%) | 11 | 19.078 Aa | 10.642 | 23.125 B | 11.895 | 24.668 b | 11.952 |
| Progressive (%) | 11 | 6.650 Aa | 4.641 | 8.348 Ba | 5.470 | 10.579 b | 6.369 |
| VCL (µm/s) | 11 | 32.132 | 8.012 | 30.210 | 7.288 | 33.721 | 7.869 |
| VAP (µm/s) | 11 | 19.779 | 7.948 | 17.738 | 7.233 | 22.441 | 8.683 |
| VSL (µm/s) | 11 | 14.812 | 7.681 | 12.935 | 6.894 | 16.641 | 7.655 |
| ALH (µm) | 11 | 1.522 | 0.220 | 1.515 | 0.202 | 1.525 | 0.159 |
| LIN (%) | 11 | 53.247 | 10.453 | 52.634 | 11.817 | 57.410 | 10.603 |
| STR (%) | 11 | 57.550 | 7.997 | 58.754 | 10.786 | 60.693 | 7.772 |
| WOB (%) | 11 | 53.247 | 10.453 | 52.634 | 11.817 | 57.410 | 10.603 |
| Live (%) | 11 | 60.185 Aa | 8.143 | 69.923 B | 7.4110 | 78.139 Cb | 6.693 |

VCL (µm/s) – velocity curvilinear, VAP (µm/s) – velocity average path, VSL (µm/s) – velocity straight line, LIN (%) – linearity (VSL/VCL), STR (%) – straightness (VSL/VAP), WOB (%) – wobble (VAP/VCL), ALH (µm) – amplitude of lateral head displacement.

A, B, C – different letters indicate statistical differences in columns ($P<0.05$).

a, b – different letters indicate statistical differences in columns ($P<0.01$).

Table 4. Sperm morphology characteristics assessed by SCA after the addition of EV proteins (mean ± SD)

| | | EV protein dose | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 µg/ml | | 25 µg/ml | | 50 µg/ml | |
| Sperm parameter | n | mean | SD | mean | SD | mean | SD |
| Abnormal acrosome | 11 | 19.492 | 6.637 | 17.932 | 7.038 | 19.081 | 8.598 |
| Abnormal angle | 11 | 1.982 | 1.901 | 1.884 | 3.228 | 2.166 | 2.045 |
| Abnormal insertion | 11 | 7.663 | 3.484 | 9.197 | 4.115 | 8.391 | 3.884 |
| Abnormal size | 11 | 15.050 | 6.668 | 17.451 | 8.436 | 14.850 | 7.356 |
| Abnormal | 11 | 18.240 | 13.786 | 22.448 | 12.829 | 20.317 | 15.454 |
| Amorphous | 11 | 27.136 | 15.109 | 26.829 | 18.269 | 24.993 | 18.588 |
| Angulation | 11 | 7.159 | 4.888 | 5.693 | 5.468 | 5.540 | 4.478 |
| Macro | 11 | 9.202 | 5.258 | 8.455 | 4.816 | 8.566 | 5.383 |
| Micro | 11 | 14.050 | 5.618 | 14.242 | 9.556 | 14.323 | 11.401 |
| Multiple | 11 | 0.086 | 0.286 | 0.089 | 0.295 | 0.065 | 0.214 |
| Normal acrosome | 11 | 80.508 | 6.637 | 82.068 | 7.038 | 80.919 | 8.598 |
| Normal midpiece | 11 | 84.015 | 6.623 | 81.855 | 81.855 | 84.143 | 7.289 |
| Normal shape | 11 | 72.255 | 15.796 | 72.245 | 18.353 | 74.309 | 18.311 |
| Normal size | 11 | 76.748 | 9.210 | 77.304 | 10.565 | 77.111 | 12.906 |
| Normal tail | 11 | 70.919 | 13.256 | 68.685 | 13.156 | 71.301 | 16.321 |
| Rolled | 11 | 2.900 | 2.161 | 2.695 | 3.292 | 2.060 | 2.016 |
| Short | 11 | 0.582 | 1.037 | 0.388 | 0.704 | 0.441 | 1.462 |
| Thin | 11 | 0.526 | 0.925 | 0.925 | 1.204 | 0.695 | 0.695 |
| Abnormal acrosome | 11 | 19.492 | 6.637 | 17.932 | 7.038 | 19.081 | 8.598 |
| Abnormal angle | 11 | 1.982 | 1.901 | 1.884 | 3.228 | 2.166 | 2.045 |

## Discussion

Cryopreservation of semen causes damage to the cell membrane of stallion sperm, which results in a decrease in sperm parameters, and, thus, the quality and suitability for insemination of semen after thawing (Bustani and Baiee, 2021). Therefore, the addition of certain additives to the diluents used in this process is still being evaluated, with the hope that they protect semen from the deleterious effects of the freezing process and improve sperm quality. Previous work has shown that the addition of EVs secreted by AT-MSCs to freezing media improved sperm motility after thawing, as well as the plasma and acrosomal membrane integrity of canine

and rat spermatozoa (Qamar et al., 2019; Mokarizadeh et al., 2013).

In this study, we investigated the effect of the addition of EVs obtained from equine MSCs isolated from adipose tissue to a semen-freezing extender on the motility, viability and morphology of stallion sperm. We used the criteria set by the ISCT (Casado-Díaz et al., 2020) and demonstrated that stem cells showed adherence and could be differentiated into adipocytes, osteocytes and chondrocytes as confirmation of their phenotype, and then the presence of specific surface markers. Using NTA analysis, the size of EV-MSCs was measured, finding an average size of 144.9±4.4 nm, which confirmed the generation of small micro-vesicles, according to the latest MISEV2018 reports (Théry et al., 2018). High-resolution flow cytometric analysis confirmed the presence of the tetraspanins CD63 and CD81 on the EV surface, which are considered exosomal markers.

In this study, we showed for the first time that the addition of EVs obtained from AT-MSCs can improve the parameters of cryopreserved stallion semen. Initially, we showed a significant decrease in the quality of semen of Hucul stallions after the cryopreservation process, which was probably largely due to the freezing after 24 hours of storage. However, a statistically significant decrease in the quality of semen of Hucul stallions was also demonstrated in earlier paper (Bugno-Poniewierska et al., 2021), which suggests that the semen of primitive horses might be more sensitive to cryopreservation damages than that of modern horse breeds. The addition of 25 μg/ml and 50 μg/ml of EV protein caused statistically significant improvement (compared to controls) of progressive motility and sperm viability, which are considered key factors in maintaining sperm function (Qamar et al., 2019). However, other parameters of sperm motility, e.g., the velocity curvilinear, velocity average path, velocity straight line, and amplitude of lateral head displacement, did not show a significant difference between those samples with the addition of EVs, and those without. The sperm morphology parameters analyzed herein showed no statistically significant differences between the samples.

The positive effect of EV supplementation on semen parameters has also been demonstrated in other animal species. The first report showing the effect of the addition of MSC-derived micro-vesicles on improving the quality of cryopreserved rat sperm was published by Mokarizadeh et al. (2013). Despite the unclear causative factor, it was shown that progressive movement and sperm viability demonstrated signi-ficant differences when 50 μg and 100 μg of micro-vesicles were added compared to controls.

Qamar et al. (2019) showed that treating canine sperm with exosomes derived from canine AT-MSCs increases their fertility and viability after thawing. The authors suggested that treatment with exosomal proteins assisted in maintaining the integrity of the plasma membrane as well as the chromatin material of canine semen during cryopreservation. Improvements in standard semen quality indices were observed in these studies, including progressive motility, lateral displacement of the sperm head (ALH), viability, membrane integrity, and testis. The authors also reported that there was an increase in ALH in their samples and no difference in linearity or rectilinearity between samples, indicating hyperactivation (Qamar et al., 2019), which is related to pregnancy rates after insemination (Johnston et al., 1994).

Other reports have also shown that treatment of cat semen with oviductal EVs resulted in improved sperm motility (Ferraz et al., 2019).

Studies in which EVs have been identified in male reproductive fluids are also available (Baskaran et al., 2020). EVs from the epididymis and prostate (epididymosomes and prostasomes, respectively) are well characterized, and their role in sperm physiology has been extensively studied (Machtinger et al., 2016). Epididymosomes are EVs that are released from epididymal epithelial cells via apocrine secretion (Machtinger et al., 2016). Furthermore, studies provide strong evidence of the involvement of EVs in sperm maturation (Tamessar et al., 2021). Conversely, prostasomes are a subclass of exosomes that originate and are secreted from the acinar epithelial cells of the prostate gland. Prostasomes play a key physiological role by interacting with sperm and enabling the transfer of molecules and proteins necessary for fertilization (Aalberts et al., 2013). Prostasomes promote sperm motility and capacitation due to the calcium content and acrosomal reaction on the way to the egg cell. The results obtained herein suggest that EVs derived from AT-MCS may perform similar functions to EVs derived from male reproductive fluids.

Stallion semen stabilizes after a month of storing in liquid nitrogen, while extracellular vesicles retain their structure and content at –80°C without losing their properties, therefore the effect of extracellular vesicles may be long-lasting. However, in order to confirm this assumption, it will be necessary to conduct future research.

Further research should also focus on examining the effect of extracellular vesicles in other breeds of horses, which would allow for generalization of the effect of these vesicles. As well, the recognition of their mechanism of action requires further research at the cellular and molecular levels.

**Conclusion**

In conclusion, the results of this study show that when added to semen, MSC-derived EVs may have a beneficial effect on improving sperm motility and viability in horses. This indicates that using EVs in addition to semen extenders may have a positive effect on the semen cryopreservation process, thus obtaining semen of better quality after thawing. Our research may be the first step in understanding the effect of EVs on stallion sperm in the cryopreservation process and the mechanism of communication between EVs and target cells. Therefore, to better understand the effects of EVs and MSCs on horse

sperm, it is planned to extend the research to genetic analysis of EV content and use other tissues as a source of stem cells.

**Acknowledgments**

We acknowledge financial support from the University of Agriculture in Krakow [grant number: SUB020013-D015].

**Author contribution statement**

Sebastian Sawicki: conceptualization, investigation, methodology, data curation, formal analysis, writing – original draft. Artur Gurgul: investigation, formal analysis, writing – review and editing. Julia Gabryś: investigation. Barbrara Kij-Mitka: investigation. Michał Bochenek: formal analysis. Elżbieta Karnas: formal analysis. Zenon Podstawski: methodology. Bogusława Długosz: methodology. Monika Bugno-Poniewierska: conceptualization, writing – review and editing, supervision, funding acquisition.

**Declaration of competing interest**

The authors declare no conflict of interest. Due to the acquisition of the research material from the slaughterhouse, the approval of the experiment by the Local Ethics Committees for Animal Experimentation in Krakow was not necessary.

**References**

Aalberts M., Stout T.A., Stoorvogel W. (2013). Prostasomes: extracellular vesicles from the prostate. Reproduction, 147: 1–14.

Alvarenga M.A., Papa F.O., Landim-Alvarenga F.C., Medeiros A.S. (2005). Amides as cryoprotectants for freezing stallion semen: a review. Anim. Reprod. Sci., 89: 105–113.

Alvarez J.G., Storey B.T. (1992). Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl., 13: 232–241.

Amann R.P., Pickett B.W. (1987). Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J. Equine Vet. Sci., 7: 145–173.

Baiee F.H., Wahid H., Rosnina Y., Ariff O., Yimer N., Jeber Z., Salman H., Tarig A., Harighi F. (2018). Impact of *Eurycoma longifolia* extract on DNA integrity, lipid peroxidation, and functional parameters in chilled and cryopreserved bull sperm. Cryobiology, 80: 43–50.

Barberini D.J., Freitas N.P., Magnoni M.S., Maia L., Listoni A.J., Heckler M.C., Sudano M.J., Golim M.A., Landim-Alvarenga F.C., Amorim R.M. (2014). Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: immunophenotypic characterization and differentiation potential. Stem Cell Res. Ther., 5: 25.

Baskaran S., Selvam M.K.P., Agarwal A. (2020). Exosomes of male reproduction. Adv. Clin. Chem., 95: 149–163.

Basu J., Ludlow J.W. (2016). Exosomes for repair, regeneration and rejuvenation. Expert. Opin. Biol. Ther., 16: 489–506.

Bourin P., Bunnell B.A., Casteilla L., Dominici M., Katz A.J., March K.L., Redl H., Rubin J.P., Yoshimura K., Gimble J.M. (2013). Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy, 15: 641–648.

Bugno-Poniewierska M., Kij-Mitka B., Podstawski Z., Długosz B., Lasek O., Mrowiec P., Tischner M. (2021). Characteristics of diluted-stored and post-thawed semen of Hutsul stallions. Acta Vet. Hung., 70: 51–57.

Bundgaard L., Stensballe A., Elbæk K.J., Berg L.C. (2018). Mapping of equine mesenchymal stromal cell surface proteomes for identification of specific markers using proteomics and gene expression analysis: an in vitro cross-sectional study. Stem Cell Res. Ther., 9: 288.

Bustani G.S., Baiee F.H. (2021). Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. Vet. World, 14: 1220–1233.

Casado-Díaz A., Quesada-Gómez J.M., Dorado G. (2020). Extracellular vesicles derived from mesenchymal stem cells (MSC) in regenerative medicine: Applications in skin wound healing. Front. Bioeng. Biotechnol., 8: 146.

Chen Y., Shao J.Z., Xiang L.X., Dong X.J., Zhang G.R. (2008). Mesenchymal stem cells: a promising candidate in regenerative medicine. Int. J. Biochem. Cell Biol., 40: 815–820.

Contreras M.J., Treulen F., Arias M.E., Silva M., Fuentes F., Cabrera P., Felmer R. (2020). Cryopreservation of stallion semen: Effect of adding antioxidants to the freezing medium on sperm physiology. Reprod. Domest. Anim., 55: 229–239.

Davis M.E. (2016). Exosomes. Circ. Res., 119: 1280–1282

de Andrade A.F.C., Arruda R.P., Torres M.A., Pieri N.C.G., Leite T.G., Celeghini E.C.C., Oliveira L.Z., Gardés T.P., Bussiere M.C., Silva D.F. (2018). Nitric oxide in frozen-thawed equine sperm: Effects on motility, membrane integrity and sperm capacitation. Anim. Reprod. Sci., 195: 176–184.

Dominici M., Le Blanc K., Mueller I., Slaper-Cortenbach I., Marini F., Krause D., Deans R., Keating A., Prockop D., Horowitz E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8: 315–317.

Ferraz M.A.M.M., Carothers A., Dahal R., Noonan M.J., Songsasen N. (2019). Oviductal extracellular vesicles interact with the spermatozoon's head and mid-piece and improves its motility and fertilizing ability in the domestic cat. Sci. Rep., 9: 9484.

Foote R.H., Leonard E.P. (1964). The influence of pH, osmotic pressure, glycine, and glycerol on the survival of dog sperm in buffered-yolk extenders. Cornell Vet., 54: 78–89.

Gabryś J., Kij-Mitka B., Sawicki S., Kochan J., Nowak A., Łojko J., Karnas E., Bugno-Poniewierska M. (2022). Extracellular vesicles from follicular fluid may improve the nuclear maturation rate of in vitro matured mare oocytes. Theriogenology, 188: 116–124.

Gangadaran P., Hong C.M., Ahn B.C. (2017 a). Current perspectives on in vivo noninvasive tracking of extracellular vesicles with molecular imaging. Biomed. Res. Int., 9158319.

Gangadaran P., Rajendran R.L., Lee H.W., Kalimuthu S., Hong C.M., Jeong S.Y., Lee S.-W., Lee J., Ahn B.-C. (2017 b). Extracellular vesicles from mesenchymal stem cells activates VEGF receptors and accelerates recovery of hindlimb ischemia. J. Control. Release., 264: 112–126.

Grzybowska A., Lorenc T., Olejarz W., Nowicka G. (2019). Egzosomy jako nośniki informacji w komunikacji między komórkami nowotworowymi (in Polish). Biul. Wydz. Farm. WUM, 2: 6–13.

Jiang W., Xu J. (2020). Immune modulation by mesenchymal stem cells. Cell Prolif., 53: 12712.

Johnston R.C., Mbizvo M.T., Summerbell D., Kovacs G.T., Baker H.W. (1994). Relationship between stimulated hyperactivated motility of human spermatozoa and pregnancy rate in donor insemination: a preliminary report. Hum. Reprod., 9: 1684–1687.

Klymiuk M.C., Balz N., Elashry M.I., Heimann M., Wenisch S., Arnhold S. (2019). Exosomes isolation and identification from equine mesenchymal stem cells. BMC Vet. Res., 15.

Lançoni R., Celeghini E.C.C., Alves M.B.R., Lemes K.M., Gonella-Diaza A.M., Oliveira L.Z., Arruda R. (2018). Melatonin added to cryopreservation extenders improves the mitochondrial membrane potential of postthawed equine sperm. J. Equine Vet. Sci., 69: 78–83.

Machtinger R., Laurent L.C., Baccarelli A.A. (2016). Extracellular

vesicles: roles in gamete maturation, fertilization and embryo implantation. Hum. Reprod., 22: 182–193.
Medina-León A.Z., Domínguez-Mancera B., Cazalez-Penino N., Cervantes-Acosta P., Jácome-Sosa E., Romero-Salas D., Barrientos-Morales M. (2019). Cryopreservation of horse semen with a liposome and trehalose added extender. Austral. J. Vet. Sci., 51: 119–123.
Meirelles S., Fontes A.M., Covas D.T., Caplan A.I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine Growth Factor Rev., 20: 419–427.
Mokarizadeh A., Rezvanfar M.A., Dorostkar K., Abdollahi M. (2013). Mesenchymal stem cell derived microvesicles: trophic shuttles for enhancement of sperm quality parameters. Reprod. Toxicol., 42: 78–84.
Nakano M., Nagaishi K., Konari N., Saito Y., Chikenji T., Mizue Y., Fujimiya M. (2016). Bone marrow-derived mesenchymal stem cells improve diabetes-induced cognitive impairment by exosome transfer into damaged neurons and astrocytes. Sci. Rep., 6: 24805.
Nouri H., Shojaeian K., Samadian F., Lee S., Kohram H., Lee J.I. (2018). Using resveratrol and epigallocatechin-3-gallate to improve cryopreservation of stallion spermatozoa with low quality. J. Equine Vet. Sci., 70: 18–25.
Oliveira R.A., Wolf C.A., Viu M.A.O., Gambarini M.L. (2013). Addition of glutathione to an extender for frozen equine semen. J. Equine Vet. Sci., 33: 1148–1152.
Ophelders D.R., Wolfs T.G., Jellema R.K., Zwanenburg A., Adriessen P., Delhaas T., Ludwig A.-K., Radtke S., Peters V., Janssen L., Giebel B., Kramer B.W. (2016). Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. Stem Cells Transl. Med., 5: 754–763.
Papa F.O., Felício G.B., Melo-Oña C.M., Alvarenga M.A., de Vita B., Trinque C., Puoli-Filho J.N.P., Dell'Aqua Jr J.A. (2011). Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. Anim. Reprod. Sci., 129: 73–77.
Pavani K.C., Hendrix A., van Den Broeck W., Couck L., Szymanska K., Lin X., Koster J.D., Soom A.V., Leemans B. (2018). Isolation and characterization of functionally active extracellular vesicles from culture medium conditioned by bovine embryos in vitro. Int. J. Mol. Sci., 20: 38.
Pillet E., Duchamp G., Batellier F., Beaumal V., Anton M., Desherces S., Schmitt E., Magistrini M. (2011). Egg yolk plasma can replace egg yolk in stallion freezing extenders. Theriogenology, 75: 105–114.
Qamar A.Y., Fang X., Kim M.J., Cho J. (2019). Improved post-thaw quality of canine semen after treatment with exosomes from conditioned medium of adipose-derived mesenchymal stem cells. Animals (Basel), 9: 865.
Tamessar C.T., Trigg N.A., Nixon B., Skerrett-Byrne D.A., Sharkey D.J., Robertson S.A., Bromfield E.G., Schjenken J. (2021). Roles of male reproductive tract extracellular vesicles in reproduction. Am. J. Reprod. Immunol., 85: 13338.
Taylor S.E., Smith R.K., Clegg P.D. (2007). Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? Equine Vet. J., 39: 172–180.
Théry C., Witwer K.W., Aikawa E., Alcaraz M.J., Anderson J.D., Andriantsitohaina R., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles, 7: 1535750.
Treulen F., Aguila L., Arias M.E., Jofré I., Felmer R. (2019). Impact of post-thaw supplementation of semen extender with antioxidants on the quality and function variables of stallion spermatozoa. Anim. Reprod. Sci., 201: 71–83.
Wu Z., Zheng X., Luo Y., Huo F., Dong H., Zhang G., Yu W., Tian F., He L., Chen J. (2015). Cryopreservation of stallion spermatozoa using different cryoprotectants and combinations of cryoprotectants. Anim. Reprod. Sci., 163: 75–81.
Yáñez-Mó M., Siljander P.R., Andreu Z., Zavec A.B., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. J. Extracell. Vesicles, 4: 27066.
Zhang Y., Yu M., Tian W. (2016). Physiological and pathological impact of exosomes of adipose tissue. Cell Prolif., 49: 3–13.

Received: 6 II 2024
Accepted: 7 VI 2024