DOI: 10.29326/2304-196X-2023-12-4-354-362

Effect of mesenchymal stem cells on animal semen during storage

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ABSTRACT

Mesenchymal stem cells (MSCs) have been known to mankind since the mid-20th century. The comprehensive study revealed their high biologically active potential. Capacity of forming several types of body tissues was demonstrated. The stem cells, like any other cells, exert their effect on surrounding cells and tissues by secreting extracellular vesicles. The extracellular vesicles of the stem cells possess biological activity of parent cells. Taking into account the regenerative potential of the mesenchymal stem cells, they are currently used in medicine, and also in veterinary medicine for treatment of various injuries of the companion animals. Effect of the mesenchymal stem cells on boar and rat sperm cells during 12-hour storage was studied. The study results demonstrated that during 12 hours of coincubation, the porcine MSCs contributed to the survival of the boar sperm cells and maintenance of their motility at 60–80% (depending on the solvent) as compared to the controls. Such a significant effect was not however observed during coincubation of the rat sperm cells with rat MSCs. But it should be noted that before the 3rd hour of coincubation, the experimental sperm motility was higher than that of the control. By hour 5 of the observation, this difference was leveled. The rat and boar sperm cells are likely to have different physiological characteristics, which were reflected in the results obtained. Therefore, possibility of using the MSCs for the storage and cryopreservation of the semen of some animals was demonstrated, but this requires further research.

Keywords: mesenchymal stem cells (MSCs), pig, boar, rat, sperm cells

Acknowledgements: The study was carried out at the expense of a grant from the Russian Science Foundation No. 23-26-00172 within the framework of the Strategic Academic Leadership Program of Kazan Federal University ("Priority-2030").

For citation: Zakirova E. Yu., Malanyeva A. G., Aimaletdinov A. M. Effect of mesenchymal stem cells on animal semen during storage. *Veterinary Science Today*. 2023; 12 (4): 354–362. DOI: 10.29326/2304-196X-2023-12-4-354-362.

Conflict of interests: The authors declare no conflict of interests.

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УДК 576.32/.36:636.082.453.53

Влияние мезенхимных стволовых клеток на сперму животных при хранении

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РЕЗЮМЕ

Мезенхимные стволовые клетки известны человечеству с середины XX в. В ходе проведенного всестороннего изучения выявлен их высокий биологически активный потенциал. Показана возможность образовывать несколько видов тканей организма. Стволовые клетки, как и любые другие, оказывают свое воздействие на окружающие клетки и ткани путем выделения внеклеточных везикул. Внеклеточные везикулы стволовых клеток обладают биологической активностью родительских клеток. Принимая во внимание регенеративный потенциал мезенхимных стволовых клеток, в настоящее время их применяют в медицине, а также в ветеринарии для лечения различных травм животных-компаньонов. Были проведены исследования по изучению влияния мезенхимных стволовых клеток на сперматозоиды хряка и крысы при хранении в течение 12 ч. Согласно полученным результатам, мезенхимные стволовые клетки свиньи способствуют выживанию сперматозоидов хряка и сохранению их подвижности на 60–80% (в зависимости от растворителя) по сравнению с контролем в течение 12 ч соинкубирования. Однако такого значительного эффекта не наблюдали при соинкубировании сперматозоидов крыс с мезенхимными стволовыми клетками крысы. Но необходимо отметить, что до 3-го ч соинкубирования подвижность сперматозоидов в опыте была выше, чем в контроле. К 5-му ч наблюдения эта разница нивелировалась. Вероятно, сперматозоиды крысы и хряка имеют различные физиологические особенности, которые отразились на полученных результатах. Таким образом, показана возможность использования мезенхимных стволовых клеток для хранения и, возможно, криоконсервации спермы некоторых животных, но для этого требуется проведение дальнейших исследований.

Ключевые слова: мезенхимные стволовые клетки, свинья, хряк, крыса, сперматозоиды

Благодарности: Исследование выполнено за счет гранта Российского научного фонда № 23-26-00172 в рамках Программы стратегического академического лидерства Казанского федерального университета («Приоритет-2030»).

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

Для цитирования: Закирова Е. Ю., Маланьева А. Г., Аймалетдинов А. М. Влияние мезенхимных стволовых клеток на сперму животных при хранении. *Ветеринария сегодня*. 2023; 12 (4): 354–362. DOI: 10.29326/2304-196X-2023-12-4-354-362.

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INTRODUCTION

Among the various practical problems of animal husbandry in agriculture, one of the most urgent ones involves the increase of the efficiency of herd reproduction by maximizing the use of high-value breeders. This is of great importance for ensuring further progress in animal husbandry. An important area for improving reproduction is the use of artificial insemination. Artificial insemination is a highly effective method of improving the pedigree and performance properties of animals by using high-value breeders. One of the determining factors of artificial insemination effectiveness is the improvement of methods for storing animal semen in a chilled or deep-frozen state. However, when the semen is being technologically processed, diluted with synthetic media and stored chilled or deep-frozen, the spermatozoa are significantly damaged structurally and physiologically. This results in the failure of the plasma membranes' permeability and release of a number of enzymes and other cell metabolic components from the spermatozoa thus leading to the considerable loss of the semen fertility [1]. The causes for the decrease in the fertility of chilled and frozen boar semen have not yet been definitively clarified. One of them is activation of lipid peroxidation processes. When the spermatozoa are cool-stored, superoxide radicals being the precursors of highly active oxygen radicals can accumulate in them. Their formation leads to the damage of the sperm DNA, proteins, lipids of the cytoplasmic membrane, etc. In this regard, researches are being conducted to improve the protective properties of synthetic diluents intended for semen of a specific animal species by introducing various natural or synthetic antioxidants, as well as other biologically active substances into their composition [2].

Development of technologies for *in vitro* storage of the semen opens up new opportunities for its use in animal reproductive medicine. This is especially relevant for artificial insemination in pig farming [3]. The use of this method allows to collect the semen sample at any convenient time in advance, as well as, if necessary, to carry out safe transportation. The main goal in the development of the semen storage technologies is to increase the duration of storage without significant loss of fertility [4].

The preservation of the rat semen is also of great importance in biology. Laboratory rats have been used in biomedical research for over 170 years. For many reasons, rats are preferred over mice in studies of physiology, neurobiology, pharmacology and behavior. The rat is the main animal model for the pharmaceutical industry due to its similarities with the humans in terms of drug binding and toxicological profiles, and almost every new medicinal product is tested in these animals [5]. This is due to the fact that rats and humans have similar genomic regions, so that breast cancer in rats, for example, is sensitive to hormones and has a greater similarity to human's in the disease stages as compared to the one in mice. Assisted reproductive technologies and genome-editing technology have facilitated the creation of the genetically modified rats worldwide. Genome-editing technology has increased the value of laboratory rats as important models for human diseases and medicinal product development in physiology and toxicology. However, not all newly bred rat breeds can reproduce naturally. Such animals are often bred through *in vitro* fertilization. Therefore, the long-term *in vitro* preservation of the rat sperm vitality is relevant [6].

Stem cells and their derivatives can act as biologically active substances that protect spermatozoa from damage. Discovery of stem cells and study of their properties expanded not only knowledge in cell biology, but also the possibilities of medicine [7, 8]. It has been shown that mesenchymal stem cells (MSCs) of an adult organism are not carcinogenic, non-immunogenic and can be used to stimulate regeneration of damaged tissues. The simplest and least traumatic way to obtain MSCs is to derive them from the adipose tissue [9, 10]. MSCs mediate their therapeutic effect by releasing biologically active molecules into the environment that are "packed" into microvesicles (MVs). The MVs, in their turn, are membrane vesicles, whose attachment or internalization to target cells results in a wide range of their epigenetic and phenotypic changes. Such regulation of physiological and pathological processes demonstrates promising non-cellular therapeutic possibilities of the MVs. Intercellular communication through vesicle secretion is currently considered a common phenomenon in all mammalian cells [11, 12].

A number of studies demonstrated that MSCs of all animals have similar biological activity, as a result of which they have found their application in veterinary medicine [13, 14, 15]. Currently, MSCs are successfully used to treat various pathological conditions in animals [16, 17].

Studies of the possible use of MSCs and their derivatives in reproductive veterinary technologies were started relatively recently. The studies have found that supplementation of the cryomedia with adipose tissue-derived MSCs increases the percentage of vital and motile canine spermatozoa after cryopreservation as compared with freezing without MSCs [18]. According to the published data,

a number of researchers have obtained good results when adding air-conditioned medium from MSCs of aminotic origin to the cryomedium. Herewith, they demonstrated that this supplement enhanced sperm motility and vitality, membrane integrity and mitochondrial activity of canine spermatozoa after defrosting. This effect was most likely mediated by natural MVs contained in large amount in the conditioned media during cell cultivation [19].

In view of the above, the effect of adipose tissue-derived MSCs on animal spermatozoa was studied. In this paper we present the results of storing boar and rat semen with the MSCs of these animals.

MATERIALS AND METHODS

Production of adipose tissue-derived MSC culture and its analysis. In rats (n = 3), subcutaneous adipose tissue was collected after decapitation in order to avoid the effect of an anesthetic on the course and results of the experiment. The incision site on the animal's abdomen was shaved, the skin was treated with alcohol. The skin incision was performed with sterile surgical instruments along the midline of the abdomen. Then subcutaneous fat was collected into a sterile container with saline solution (PanEco, Russia) supplemented with 1% of penicillin-streptomycin (PanEco, Russia).

A fragment of the subcutaneous adipose tissue was collected from pigs (n = 3) into a sterile container under general anesthesia in the veterinary operating room.

The adipose tissue samples were transported at max 15 °C for 2–4 hours after their collection. The cells were isolated in sterile conditions of the cell laboratory according to the previously described standard procedure [20]. The stem cell-containing stromal vascular fraction was isolated as follows: the adipose tissue was mechanically crushed with sterile scissors into fragments of about 1 mm^3 and incubated in the crab collagenase solution (BioloT, Russia) at a final concentration of 0.2% for 1 hour at 37 °C while rocking on the shaker. The resulted cell suspension was precipitated by centrifugation at 1,500 rpm for 10 minutes. The supernatant was removed. Then the deposited cells were washed: the precipitate was resuspended three times in 0.9% NaCl solution (PanEco, Russia) and precipitated by centrifugation at 1,500 rpm for 10 minutes. The obtained cells were cultured in α-MEM medium (PanEco, Russia) containing 10% blood fetal bovine serum – FBS (PanEco, Russia), 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (PanEco, Russia). After 24 hours, the nutrient medium was replaced with a fresh growth medium. At the same time, cells that were not attached to the plastic culture flask were removed. The growth medium was subsequently changed once every 3 days. As soon as the monolayer reached 80% density, the MSCs were reseeded. The cells were reseeded by trypsinization using 0.25% trypsin-EDTA solution (PanEco, Russia). Cells of passages 4–5 were further used. This is due to the fact that the MSCs of passage 3 are already evaluated as homogeneous and stable populations, and the cells of passage 8 are aging cells beginning to show signs of genetic instability and decrease in differentiation potential.

To perform flow cytofluorometry, the MSCs were removed from the plastic culture flask by trypsinization. They were further washed with Dulbecco sodium phosphate buffer solution – DPBS (PanEco, Russia) by centrifugation for 5 minutes at 1,500 rpm and fixed with 4% formalin solution for 20 minutes at room temperature. After that, they were washed with DPBS, resuspended and the aliquots were stained with antibodies (AT) according to the manufacturer's instructions: monoclonal PE/Cyanine7 anti-mouse Thy-1.1 AT (Biolegend, USA); monoclonal PE anti-mouse/rat CD29 AT (Biolegend, USA); monoclonal PerCP/Cyanine5.5 anti-mouse CD73 AT (Biolegend, USA); CD34 Alexa Fluor 647 monoclonal mouse AT (Santa Cruz Biotechnology, USA). The cells were analyzed for the presence of the stem cell membrane markers using flow cytofluorimeter BD FACSAria™ III (BD Biosciences, USA). The flow cytometry result is expressed as a percentage of the total number of cells in the sample (at least 100 ths cells/aliquot).

MSCs differentiation. To study the differentiation capacities of the obtained cell cultures, the passage 4 cells were seeded on 12-well plates at 30 ths cells/well and incubated in the growth medium until the monolayer was formed. In order to induce the differentiation, the cell cultures were subsequently incubated with special media. Differentiation was carried out in three directions: osteogenic, adipogenic and chondrogenic. The differentiation results were recorded using an inverted AxioObserver Z1 microscope (Carl Zeiss, Germany).

For osteogenic differentiation, α-MEM medium was used, which was supplemented with 10% FBS, 100 nM dexamethasone (Sigma, USA), 0.5 µM ascorbic acid 2-phosphate (Sigma, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (PanEco, Russia). α-MEM medium supplemented with 10% FBS, 0.5 µM ascorbic acid 2-phosphate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine was used as a control medium. For osteogenic differentiation, 0.2 µM of β-glycerophosphate solution (Sigma, USA) was added to the medium as well as to the control medium from day 10 of incubation. The media were changed every three days.

Von Kossa staining technique was used to determine mineralization, which is a sign of osteogenic differentiation. This reaction is based on the binding of silver ions to phosphate groups. The resulted compound undergoes photochemical degradation with the release of silver ions, staining the mineral deposits gray-brown. For this purpose, the nutrient medium was removed from the wells of the plate before staining. The cells were washed with 0.9% NaCl solution and fixed with 4% formalin solution for 30 minutes at room temperature. Then the wells were threefold thoroughly washed with a sufficient amount of distilled water and filled with a 2% silver nitrate and distilled water solution. The plates were incubated in the dark for 10 minutes. Then they were washed with distilled water and incubated in bright light for 1 hour.

To induce adipogenic differentiation, DMEM High glucose medium (PanEco, Russia) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 µM dexamethasone, 100 µM indomethacin (Sigma, USA), 500 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma, USA) and 10 ug/mL insulin (Sigma, USA) was used. From day 10, the medium was replaced with a maintenance one free from dexamethasone, indomethacin and IBMX. At all stages DMEM High glucose supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine was used as a control medium. The media were changed every three days.

Qualitative staining of lipid inclusions with Sudan-3 stain (Sigma, USA) was used for adipogenic differentiation. For this purpose, the nutrient medium was removed from the cell culture and fixed with a 4% formalin solution for 30 minutes at room temperature. Before staining, the wells were threefold thoroughly washed with a sufficient amount of distilled water for 5 minutes.

When staining the cells with Sudan-3, their nuclei were additionally stained with hematoxylin and eosin. Sudan-3 stain was prepared by dissolving 0.02 g of powder in 10 mL of 70% ethanol. The mixture was incubated for 2 hours at 58 °C. The solution was subsequently filtered and the samples were stained at room temperature for 15–30 minutes. Then they were washed with 0.9% NaCl solution and stained with hematoxylin and eosin. Hematoxylin and eosin solutions were prepared and the cell cultures were stained according to the standard procedure.

For chondrogenic differentiation, 9×10^5 of passage 3–6 cells were taken and the same number was taken for control. They were washed from the nutrient medium and precipitated. MSCs for differentiation were resuspended with 90 µL of the chondrogenic differentiation medium, the same number of cells were resuspended in 90 µL of the control medium. The experimental and control suspensions were applied by drops at 10 µL for each of the 3 wells of a 24-well plate. MSCs were incubated at 37 °C for 2 hours for cell adhesion, after which 500 µL of the appropriate medium was added to each well. *α*-MEM supplemented with 10% FBS, 0.5 µM ascorbic acid 2-phosphate (Sigma, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine was used as a control medium. DMEM-High glucose medium supplemented with 10% FBS, 0.5 µM ascorbic acid 2-phosphate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1× ITS (Sigma, USA), 100 µM/L sodium pyruvate (PanEco, Russia), 1 µM dexamethasone (Sigma, USA), 0.5 µM ascorbic acid 2-phosphate (Sigma, USA), 10 ng/mL TGF-*β*1 (Sigma, USA) was used as a chondrogenic differentiation medium. The media were changed every three days.

For chondrogenic differentiation identification, the staining with Alcian Blue stain (Sigma, USA) was performed for the detection of acidic mucopolysaccharides being the marker of chondrogenation. For this purpose, the cell cultures were fixed in 95% ethanol and then in 70% ethanol for 5 minutes in each. After that, the cell cultures were threefold washed with distilled water for 30 seconds and stained with Alcian Blue solution for 1 hour. Then they were washed under running water for 2 minutes. Distilled water was poured over the samples and the samples were examined through the inverted visible light microscope. The Alcian Blue stain solution was prepared by dissolving 1 g of the powder in 100 mL of 0.1 M HCl.

Obtaining and analysis of animal sperm. The epididymides were derived from healthy male Wistar rats ($n = 5$) after decapitation, and from boars ($n = 3$) – immediately after slaughter. The epididymides were aseptically excised and transferred in a sterile Petri dish. The semen derived from one excised epididymis was washed with heated citrate buffer (Diaem, Russia), from the other – with DPBS in the ratio of 1:2. In case the semen was rich in spermatozoa, it was diluted 10-fold. All studies of the collected samples were carried out under the light microscope on slides heated to 37 °C. The samples meeting the standards were selected for further experiments [21].

To determine the content of pathological forms of spermatozoa, 0.05% eosin stain solution (PanEco, Russia) was added to a drop of semen on the slide in the ratio of 1:2. The semen and stain mixture was left for 3–5 minutes, after which three smears were made and visualized using the immersion microscopy; 100–200 cells were counted, identifying pathological forms and determining their percentage. The number of live (unstained) and dead (stained) spermatozoa was simultaneously calculated. Normally, the vital cells in the sample should amount to at least 58%. When assessing motility, the attention was paid to morphological defects such as droplets or curved tails. The number of sperm cells with abnormal morphology should not exceed 10%.

To determine the motility, the semen was diluted 20-fold with a heated citrate buffer/DPBS solution using semi-automatic dispensers. The number of forward-moving spermatozoa was calculated among 2 hundred counted spermatozoa, and percentage of progressive motility was determined. Ejaculates with the motility below 70% and high content of agglutinated cell groups were rejected. The calculation was performed in at least four different fields. All results were presented as a percentage of the total number of spermatozoa.

The integrity of the acrosomes in semen was determined by staining the samples with Coomassie G250 stain (Sigma, USA). The semen samples were placed on slides and incubated in a freshly prepared stain solution (0.22% Coomassie Blue G250, 50% methanol, 10% glacial acetic acid, 40% water) for 2 minutes. After that, the slides were thoroughly washed with distilled water to remove excess staining and examined under the light microscope.

The MSCs and spermatozoa were coincubated in CO_2 incubator at 37 °C for 12 hours in the ratio of 1 million rat/pig MSCs per 5 million rat/pig spermatozoa in 1 mL of citrate buffer/DPBS. The control semen samples were incubated at the concentration of 5 million germ cells per 1 mL of citrate buffer/DPBS without MSCs. The number of live and dead spermatozoa, their motility, as well as integrity of the acrosomes were determined in 3, 5 and 12 hours after the start of the experiment.

Compliance with ethical standards. The permission of the local Ethics Committee of Kazan Federal University (No. 1 of 23 February 2015 for research on the subject Genetic and cell therapy in regenerative veterinary medicine) was obtained to carry out procedures for the collection of adipose tissue.

Processing the results. Statistical processing of the obtained data was carried out by primary statistical analysis tools using Excel 2016 software. The results are presented as the arithmetic mean of the sample ± standard deviation. Secondary statistical data processing was performed using the nonparametric Mann – Whitney U-test. The differences were considered significant at a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The cells isolated from the animal adipose tissue adhered to the plastic culture flask and had a fibroblast-like morphology (Fig. 1).

The isolated cells expressed MSC markers: Thy-1, CD29, CD73 and did not express the hematopoietic stem cell marker CD34 (Table 1).

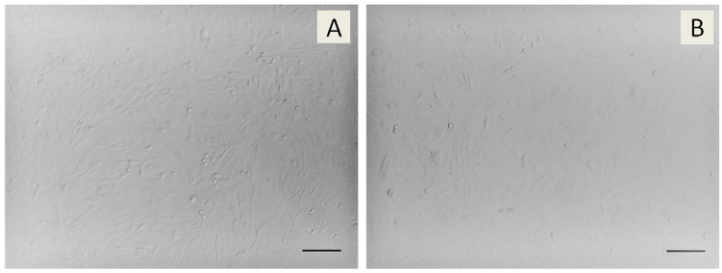

Fig. 1. Primary MSC culture: A – pigs; B – rats (100× magnification)

The results of differentiation of isolated rat and pig cells in 3 directions are shown in Figure 2.

Thus, the affiliation of isolated and cultured pig and rat cells to the MSC pool is confirmed by the typical morphology at *in vitro* growth, presence of specific MSC markers and lack of markers of other stem cells, as well as their differentiation in 3 directions.

At the next stage, 23 ± 1 and 160 ± 3 million spermatozoa/mL were obtained from rat and boar epididymides, respectively. Results of coincubation of rat/boar spermatozoa with rat/pig MSCs are shown in Figure 3.

Table 1
Number of animal MSCs ($n = 3$) in the isolated cell population demonstrating typical markers of MSC, %

Marker / Animal	Thy-1	CD29	CD73	CD34
Rat	95 ± 3	97 ± 1	95 ± 6	0
Pig	98 ± 2	94 ± 3	92 ± 4	0

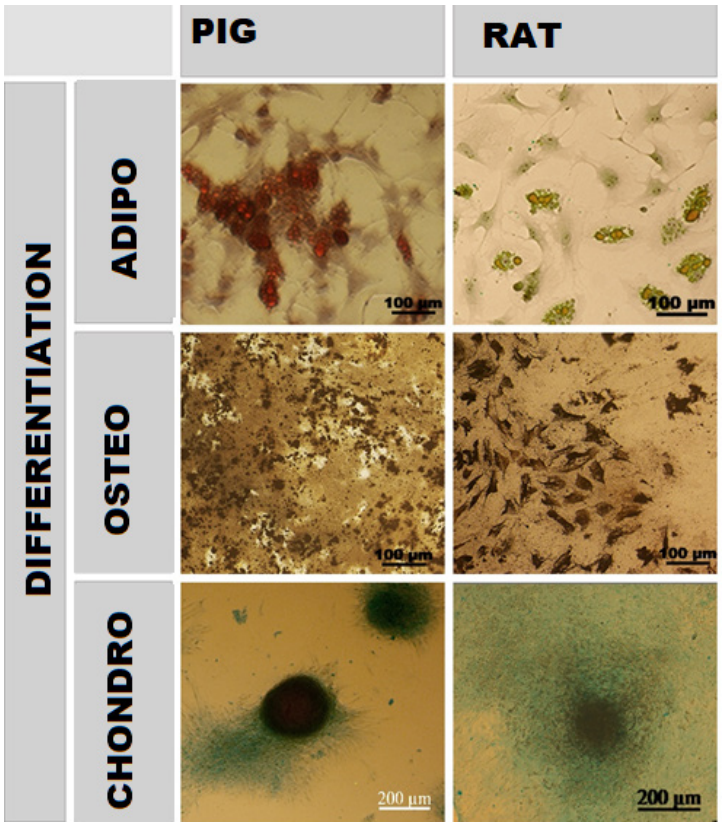

Fig. 2. Differentiation of rat and porcine MSCs of adipogenic origin (magnification is indicated in the pictures)

The vitality and progressive motility of spermatozoa are considered to be the key factors that can affect the rate of both artificial and natural insemination. The semen evaluation based on these parameters is still the gold standard for *in vitro* and *in vivo* fertility prediction [22]. According to the data obtained, the rat spermatozoa are more sensitive to storage in citrate buffer and DPBS as compared to boar spermatozoa. At the same time, they practically lose their motility after 3 hours of storage in DPBS and citrate buffer solutions, both with and without MSCs. Rat spermatozoa retained the same vitality for 5 hours, regardless of what solution they were in, while boar spermatozoa better retained their vitality and motility in solutions with MSCs. Herewith, the minimal loss of motility and the maximal number of live boar spermatozoa were recorded when stored in DPBS with MSCs as compared to citrate buffer with MSCs. But there is a significant positive effect of the presence of MSCs in the storage medium as compared to the solutions without MSCs: by hour 12 of the experiment, 18% of vital boar spermatozoa were in the citrate buffer, and when MSCs were added to the citrate buffer, the vitality amounted to 88%. When stored in DPBS, by hour 12, there were 6% of vital spermatozoa in the samples, and 88% in the medium with MSCs.

The data obtained on the change in the integrity of acrosomes throughout the whole experiment indicated that they remained practically intact in rat spermatozoa by hour 12 of incubation. Moreover, introduction of MSCs into the incubation medium had a positive effect on their integrity. In boars, breakdown of acrosomes occurred already by hour 3 of incubation in both control and experimental samples. Herewith, adding MSCs to the solution also had a protective effect on preserving the integrity of boar spermatozoa (Table 2).

It is known that in order to preserve *in vitro* motility and vitality of boar spermatozoa, the researchers recommend adding various substances to the diluent, for example, cresacin biostimulator. It has a positive effect on the motility of germ cells and belongs to organic chemical compounds [23]. However, addition of MSCs may have a positive effect not only on the spermatozoa themselves, but also on sows during insemination, since, according to the published data, the addition of the MSC conditioned medium to stallion semen samples not only did not worsen the semen parameters during 2 hours of incubation, but also mitigated early inflammatory endometrial reactions in mares during artificial insemination with these samples. The researchers associate the obtained effect with MVs extracted by the MSCs in the culture medium [24].

Our studies have shown absence of both pronounced positive and negative effects of MSCs on the rat semen as compared with the boar spermatozoa. This can be explained by the fact that, according to the published data [25], there are differences in the content of organic compounds, in particular proteins and their compounds, in the cell membrane of rat and boar spermatozoa (Table 3), as a result of which the germ cells of these animals react differently to signals from the outside.

For example, concentrations of total cholesterol of phospholipids, desmosterol, and phosphatidylethanolamine in the cytoplasmic membrane of rat and boar caudal spermatozoa are almost the same. But the content

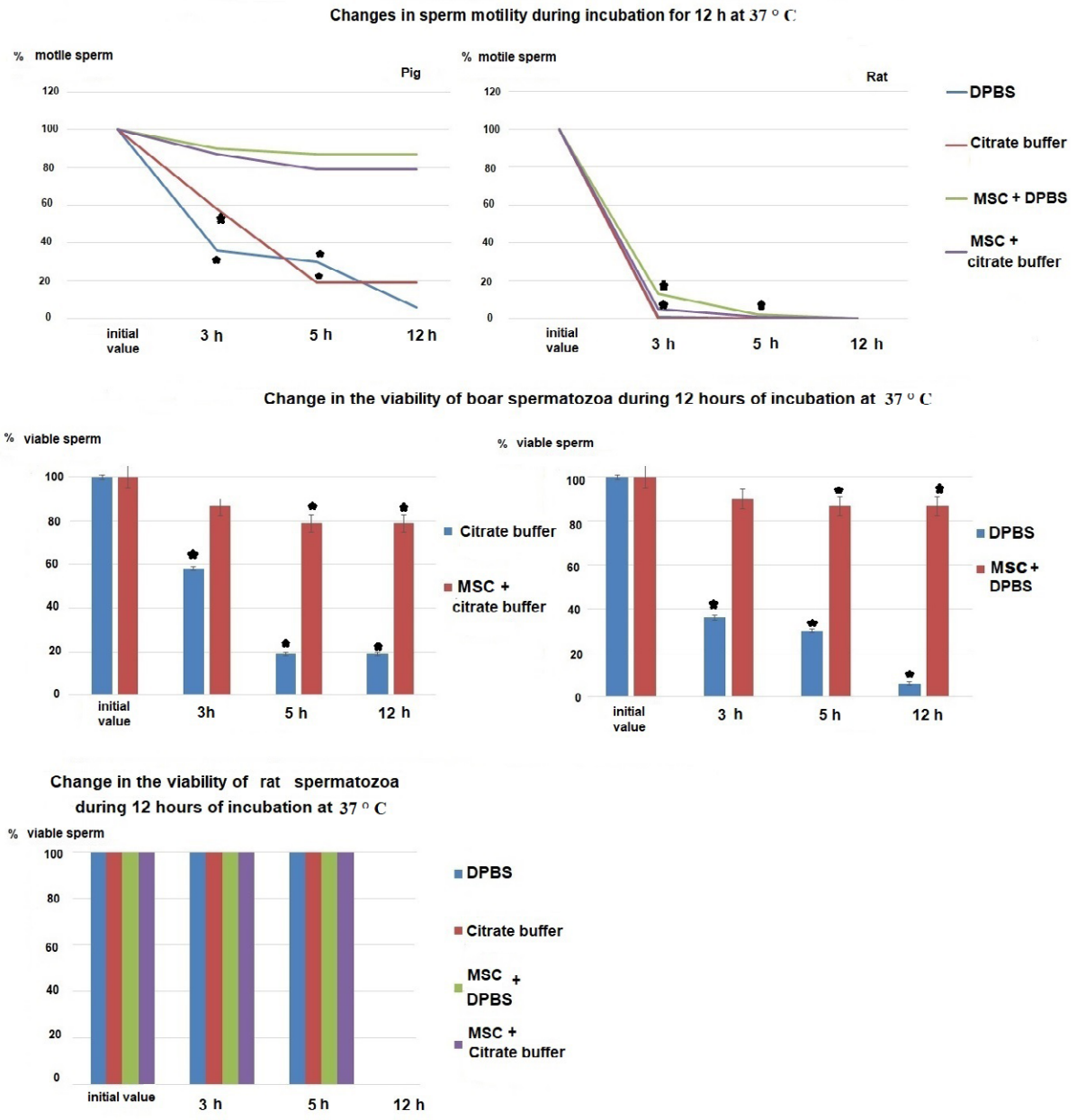

Fig. 3. Measuring vitality and motility of the boar and rat sperm cells (p ≤ 0.05 as compared to initial group value)*

of sphingomyelin and phosphatidylserine varies significantly. The amount of sphingomyelin in the cytoplasmic membrane of rat caudal spermatozoa is nearly two-times lower than in boar spermatozoa. It is well known that sphingomyelin has an effect on spermatozoa motility and integrity of their membranes. There is evidence of positive correlation between the content of this phospholipid in the cell membrane of human spermatozoa and their motility [26]. Rat spermatozoa contain less sphingomyelin as compared to boar spermatozoa. That is probably why they lose motility in the control sample already by hour 3 of incubation. But even under these conditions, rat spermatozoa retain greater motility with MSCs (Fig. 2) as compared to the control group. Different content of proteins responsible for the motion activity of spermatozoa in the membrane of boar and rat male germ cells, and, as a result, different performance, are most likely due to different length of the genital tract in females of these animals.

Phospholipid phosphatidylserine concentrations also differ significantly in boar and rat spermatozoa. It is in charge of the activity of spermatozoa. This phospholipid is normally located on the inner side of the cell membrane, and when transferred to the outer side as a result of various processes in the cell, it is an early marker of apoptosis [27]. In their article, P. Agrawal et al. [25] determined concentration of phosphatidylserine in the whole membrane of spermatozoa. Therefore, it is impractical to draw any conclusions from these data regarding the work carried out.

It is also known that rat spermatozoa are extremely sensitive to centrifugation, pipetting and cooling conditions due to their long tail, shape, head size and membrane composition [28]. Lack of any effect on rat spermatozoa during the experiment may be explained by the small number of MVs isolated from the MSCs added to the storage medium. Mokarizadeh A. et al. [29] in their studies demonstrated the data on cryopreservation of rat spermatozoa with MVs collected during the cultivation

Table 2
Changes in rat and boar acrosome integrity during the experiment, %

	Diluent		Original	3 hours	5 hours	12 hours
Rat	Citrate buffer	control	0	0	0	0.71
		adding MSCs	0	0	0	0.33*
	DPBS	control	0	0	0.31	2.00
		adding MSCs	0	0	0	0.33*
Boar	Citrate buffer	control	0	0.33	0.67	1.20
		adding MSCs	0	0.33	0.71	1.10
	DPBS	control	0	0.67	0.82	0.97
		adding MSCs	0	0.33*	0.51*	0.67*

* $p \leq 0.05$ as compared to the group control value at the given time point.

Table 3
Cytoplasmic membrane composition of rat and boar caudal sperm cells (according to P. Agrawal, 1988), μg

Substance		Rat	Boar
Phospholipid protein		0.63	1.47
Total cholesterol of phospholipids		0.18	0.17
Desmosterol		0.32	0.32
Phospholipids	phosphatidylserine	10	3
	phosphatidylethanolamine	31	28
	sphingomyelin	30	62
	diphosphate diglycerol	1	–
	other	8	7

of adipose tissue-derived MSCs. The researchers noted that addition of 25 μg of MVs based on total protein does not have any significant effect on spermatozoa as compared to the control without MVs. At the same time, cryopreservation of rat spermatozoa with 50 and 100 μg of MVs based on total protein significantly increases the number of vital and motile spermatozoa after defrosting. Unfortunately, the task of this work was not to isolate and determine the effect of MSCs derivatives on the state of rat spermatozoa, so we cannot reliably confirm this assumption.

CONCLUSION

In the last few decades, the majority of the research has been focused on the methods aimed to improve the efficiency of the semen storage/cryopreservation, which is considered one of the main problems in reproductive biotechnology. The applied approaches are based on the protection of spermatozoa from the harmful effects of storage and cryopreservation procedures, including use of various extenders, cryoprotectors, antioxidants and nutritional components. Moreover, some of these studies have focused on repair damaged sperm during the freeze-thaw process [30]. Repair of the spermatozoa damaged during storage is considered extremely important for improving their vitality and fertility [31]. It is known that stem cells excrete paracrine factors that enhance cellular protection and trigger anti-apoptotic and antioxidant mechanisms [32]. And stem cell derivatives, MVs, have effects similar to their parent cells on the surrounding tissues and cells [33]. The use of the stem cells and their derivatives (MVs) is likely to protect semen from the negative effects of storage/cryopreservation, such as oxidative stress, apoptosis, DNA damage and loss of mitochondrial activity. When analyzing the results obtained, it is impossible to unambiguously recommend the use of MSCs in the storage of spermatozoa of all animals. It is necessary to clarify the features of the interaction between animal spermatozoa and MSCs, mechanism of such effects and features of artificial insemination of animals with such semen. This will allow filling in serious gaps in knowledge and technologies for long-term storage of germ cells. This research area is of great importance for animal science and biotechnology as for preservation of germ cells of various species of rare and endangered animals. Along with scientific progress related to the discovery of sperm fertility markers, there is an urgent need to improve vitality and fertility of spermatozoa after storage (thawing) by improving storage methods (cryopreservation) in order to obtain high-performance farm animals to ensure global food security.

REFERENCES

1. Epishina T. M. Efficiency of estrum stimulation at pigs by physical factors. *Zootechniya*. 2009; 2: 29–30. EDN: JXDGUD. (in Russ.)

2. Erokhin A., Epishina T., Fedina N. Vlijanie bych'ego syvorotochnogo al'bumina na fertil'nost' ohlazhdennogo semeni hrjakov = Effect of bovine serum albumin on the fertility of chilled boar sperm. *Pigbreeding*. 2007; 4: 26–27. EDN: IBOJAR. (in Russ.)

3. Medvedev G. F., Gavrichenko N. I., Budevich A. I. Dilutings for average-time keeping of the semen boars. *Aktual'nye problemy intensivnogo razvitiya zhivotnovodstva*. 2011; 14 (2): 314–320. EDN: RUHIEX. (in Russ.)

4. Isaev D. A., Zakharova E. E., Kapralova I. V., Krivokharchenko I. S., Kartavenko T. V., Mikhaylovskaya G. V., et al. Short-term liquid sperm storage in IVF/ICSI cycles. *Russian Journal of Human Reproduction.* 2015; 21 (4): 65–70. DOI: 10.17116/repro201521465-70. (in Russ.)

5. Meek S., Mashimo T., Burdon T. From engineering to editing the rat genome. *Mammalian Genome*. 2017; 28 (7–8): 302–314. DOI: 10.1007/s00335-017-9705-8.

6. Neff E. P. Rats on the rise. *Lab Animal (NY)*. 2021; 50 (8): 205–208. DOI: 10.1038/s41684-021-00812-0.

7. Zakirova E. Yu., Valeeva A. N., Aimaletdinov A. M., Nefedovskaya L. V., Akhmetshin R. F., Rutland C. S., Rizvanov A. A. Potential therapeutic application of mesenchymal stem cells in ophthalmology. *Experimental Eye Research*. 2019; 189:107863. DOI: 10.1016/j.exer.2019.107863.

8. Jiménez A. J. V., Guerrero F. F. Células madre mesenquimales como nueva terapia en dermatología: conceptos básicos. *Revista Clínica de Dermatología Veterinaria*. 2017; 9: 8–18. Available at: https://immunestem.com/wp-content/uploads/2021/02/18.-TERAPIA-CELULAR-EN-DERMATOLOGIA.pdf. (in Spanish)

9. Salikhov R. Z., Masgutov R. F., Chekunov M. A., Tazetdinova L. G., Masgutova G., Teplov O. V., et al. The stromal vascular fraction from fat tissue in the treatment of osteochondral knee defect: case report. *Frontiers in Medicine. (Lausanne)*. 2018; 5:154. DOI: 10.3389/fmed.2018.00154.

10. Wang Y., Yi H., Song Y. The safety of MSC therapy over the past 15 years: a meta-analysis. *Stem Cell Research*. 2021; 12 (1):545. DOI: 10.1186/s13287-021-02609-x.

11. Zakirova E. Yu., Aimaletdinov A. M., Malanyeva A. G., Rutland C. S., Rizvanov A. A. Extracellular vesicles: new perspectives of regenerative and reproductive veterinary medicine. *Frontiers in Veterinary Science*. 2020; 7:594044. DOI: 10.3389/fvets.2020.594044.

12. Qamar A. Y., Hussain T., Rafique M. K., Bang S., Tanga B. M., Seong G., et al. The role of stem cells and their derived extracellular vesicles in restoring female and male fertility. *Cells*. 2021; 10 (9):2460. DOI: 10.3390/cells10092460.

13. Gade N. E., Nath A., Pratheesh M. D., Dubey P. K., Amarpal, Kumar G. S., Sharma G. T. Stem cell therapy in animal sciences – a review. *Agricultural Reviews*. 2012; 33 (2): 150–158. Article ID: ARCC575.

14. Bogers S. H. Cell-based therapies for joint disease in veterinary medicine: what we have learned and what we need to know. *Frontiers in Veterinary Science*. 2018; 5:70. DOI: 10.3389/fvets.2018.00070.

15. Zakirova E. Yu., Aimaletdinov A. M., Tambovsky M. A., Rizvanov A. A. Comparative characteristics of mesenchymal stem cell lines from different animal species. *Tsitologia*. 2021; 63 (2): 139–146. DOI: 10.31857/S0041377121020097. (in Russ.)

16. Dias I. E., Pinto P. O., Barros L. C., Viegas C. A., Dias I. R., Carvalho P. P. Mesenchymal stem cells therapy in companion animals: useful for immune-mediated diseases? *BMC Veterinary Research*. 2019; 15 (1):358. DOI: 10.1186/s12917-019-2087-2.

17. Naumenko E., Zakirova E., Guryanov I., Akhatova F., Sergeev M., Valeeva A., Fakhrullin R. F. Composite biodegradable polymeric matrix doped with halloysite nanotubes for the repair of bone defects in dogs. *Clays Clay Minerals*. 2021; 69: 522–532. DOI: 10.1007/s42860-021-00152-7.

18. Qamar A. Y., Fang X., Kim M. J., Cho J. Improved viability and fertility of frozen-thawed dog sperm using adipose-derived mesenchymal stem cells. *Scientific Reports*. 2020; 10 (1):7034. DOI: 10.1038/s41598-020-61803-8.

19. Mahiddine F. Y., Kim J. W., Qamar A. Y., Ra J. C., Kim S. H., Jung E. J., Kim M. J. Conditioned medium from canine amniotic membrane-derived mesenchymal stem cells improved dog sperm post-thaw quality-related parameters. *Animals (Basel)*. 2020; 10 (10):1899. DOI: 10.3390/ani10101899.

20. Aleksandrova N. M., Aimaletdinov A. M., Malanyeva A. G., Tambovskii M. A., Rizvanov A. A., Zakirova E. Yu. Features of induction of osteodifferentiation *in vitro* adipose-derived mesenchymal stem cells. *Biotekhnology*. 2022; 38 (4): 72–79. DOI: 10.56304/S0234275822040032. (in Russ.)

21. Komlatsky V. I., Velichko L. F., Velichko V. A. Biology and ethology of swine: study guide. Krasnodar: FSBEI HE Kuban SAU; 2017. 130 p. (in Russ.)

22. Simon L., Lewis S. E. M. Sperm DNA damage or progressive motility: which one is the better predictor of fertilization *in vitro*. *Systems Biology in Reproductive Medicine*. 2011; 57 (3): 133–138. DOI: 10.3109/19396368.2011.553984.

23. Glivanskaya O. I., Bogdanovich D. M. Dependence of semen quality on concentration of biostimulant in diluent for technology of artificial insemination of pigs. *Tauride Scientific Observer*. 2016; 5–2 (10): 199–202. EDN: WCKXRP. (in Russ.)

24. Tongu E. A. O., Segabinazzi L. G. T. M., Alvarenga M. L., Monteiro A., Papa F. O., Alvarenga M. A. Allogenic mesenchymal stem cell-conditioned medium does not affect sperm parameters and mitigates early endometrial inflammatory responses in mares. *Theriogenology*. 2021; 15 (169): 1–8. DOI: 10.1016/j.theriogenology.2021.03.019.

25. Agrawal P., Magargee S. F., Hammerstedt R. H. Isolation and characterization of the plasma membrane of rat cauda epididymal spermatozoa. *Journal of Andrology*. 1988; 9 (3): 178–189. DOI: 10.1002/j.1939-4640.1988.tb01031.x.

26. Shan S., Xu F., Hirschfeld M., Brenig B. Sperm lipid markers of male fertility in mammals. *International Journal of Molecular Sciences*. 2021; 22 (16):8767. DOI: 10.3390/ijms22168767.

27. Kotwicka M., Jendraszak M., Skibinska I., Jedrzejczak P., Pawelczyk L. Decreased motility of human spermatozoa presenting phosphatidylserine membrane translocation-cells selection with the swim-up technique. *Human Cell*. 2013; 26 (1): 28–34. DOI: 10.1007/s13577-011-0024-1.

28. Varisli O., Agca C., Agca Y. Short-term storage of rat sperm in the presence of various extenders. *Journal of the American Association for Laboratory Animal Science*. 2013; 52 (6): 732–737. PMID: 24351761.

29. Mokarizadeh A., Rezvanfar M. A., Dorostkar K., Abdollahi M. Mesenchymal stem cell derived microvesicles: trophic shuttles for enhancement of sperm quality parameters. *Reproductive Toxicology*. 2013; 42: 78–84. DOI: 10.1016/j.reprotox.2013.07.024.

30. Saadeldin I. M., Khalil W. A., Alharbi M. G., Lee S. H. The current trends in using nanoparticles, liposomes, and exosomes for semen cryopreservation. *Animals (Basel)*. 2020; 10 (12):2281. DOI: 10.3390/ani10122281.

31. Qamar A. Y., Fang X., Kim M. J., Cho J. Improved post-thaw quality of canine semen after treatment with exosomes from conditioned medium of adipose-derived mesenchymal stem cells. *Animals*. 2019; 9 (11):865. DOI: 10.3390/ani9110865.

32. Ferro F., Spelat R., Shaw G., Duffy N., Islam M. N., O'Shea P. M., et al. Survival/adaptation of bone marrow-derived mesenchymal stem cells after long-term starvation through selective processes. *Stem Cells*. 2019; 37 (6): 813–827. DOI: 10.1002/stem.2998.

33. Bahr M. M., Amer M. S., Abo-El-Sooud K., Abdallah A. N., El-Tookhy O. S. Preservation techniques of stem cells extracellular vesicles: a gate for manufacturing of clinical grade therapeutic extracellular vesicles and long-term clinical trials. *International Journal of Veterinary Science and Medicine.* 2020; 8 (1): 1–8. DOI: 10.1080/23144599.2019.1704992.

Received 22.10.2023
Revised 20.11.2023
Accepted 29.11.2023

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