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AUTOLOGOUS STEM CELLS THERAPY IN HORSES AND DOGS WITH CHRONIC OSTEOARTHRITIS

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Abstract. *One of the fastest growing areas of veterinary medicine over the past decade has been and still is the regenerative medicine. It focuses on the reconstruction of damaged tissues/organs to restore their physiological and functional performance. The aim of study was the use of cells called mesenchymal stem cells (MSC) as an alternative treatment method of osteoarthritis. Twelve dogs have been chosen for experiment aged 5 to 11 years. All animals were diagnosed with the degeneration of the elbow joint. The dogs were divided into 2 groups: 8 dogs formed the test group and the remaining 4 dogs formed the control group. The dogs from the first group were treated with autologous stem cells. The dogs in the control group received non-steroidal anti-inflammatory drugs (NSAIDs) – mavacoxib for a period of 6 months. Clinical examination on the first day of the experiment showed signs of lameness of the 3rd and 2nd degree in all 12 dogs. The clinical tests performed on day 180 in the control group showed signs of gait disturbance at the unchanged level. The dogs undergoing treatment with the use of autologic stem cells did not show any movement disorders. The obtained results allow drawing the conclusion that the combination of the lowest concentrations of metamizol may significantly increase the cytophysiological activity of the stem cells accompanied by the lack of morphological changes. The obtained data can be a valuable source of information for practicing veterinary doctors who introduce stem cells to treat musculoskeletal system diseases in both small and large animals.*

Keywords: *stem cells, mesenchymal stem cells (MSC), osteoarthritis, non-steroidal anti-inflammatory drugs (NSAIDs), mavacoxib*

Introduction & Analysis of recent researches and publications

One of the fastest growing areas of veterinary medicine over the past decade has been and still is the regenerative medicine. It focuses on the reconstruction of damaged tissues/organs to restore their performance – not only physiological, but also functional (Mason & Dunnill, 2008; Ratajczak et al., 2013). For this purpose somatic stem cells isolated from mature

tissues are used more and more frequently (Minguell et al., 2001; Mason & Dunnill, 2008; Zhu et al., 2008; Lai et al., 2011; Dimitrieva et al., 2012). Subsequently, the somatic cells reprogrammed into stem cells, referred to as Induced Pluripotent Stem Cells (iPSCs), are not taken into account in clinical use at this stage of the research due to their teratogenic nature. The population of stem cells which is the most frequently used in the context of veterinary medicine are Mesenchymal Stem Cells (referred to as MSC) isolated from fat (Eng. Adipose Stromal Cells – ASCS) as well as those isolated from the bone marrow (Eng. Bone Marrow Mesenchymal stem cells – BMSCs). Both mentioned populations of stem cells are multipotent cells, which means that in in vitro conditions they may differentiate into bone tissue, cartilage and adipose tissue (Barry & Murphy, 2004). Their indispensable and unique features are the ability to self-regenerate and the ability to divide up to thirty passages (Zuk et al., 2002; Zhu et al., 2008). This demonstrates their high cyto-physiological activity and ability to multiply extra-corporeally. In in vitro culture conditions, these cells are characterized by high adhesion and proliferation capacity, which allows their use not only for purely medical purposes, but also allows for breeding various kinds of implant material. The capacities of adhesion, proliferation, and differentiation of stem cells in in vitro conditions have become the basis for investigators to consider their potential application in the treatment of bone tissue and cartilage defects (Strem et al., 2005). Moreover, stem cells are of immunomodulatory nature and as it has been discovered recently they act as a nutrient for e.g. bone marrow stroma. The process of regenerating the damaged tissues, e.g. bone or cartilage tissues using stem cells can be carried out through both autocrine and paracrine signaling. As has been described recently, the stem cells can synthesize and produce Microvesicles (MVs) into the damaged tissue, which first improves communication between the cells (called Inter cellular signaling – ICS). The mesenchymal cells synthesize and produce stem microvesicles which contain a number of growth factors including insulin-like growth factor, fibroblast growth factor, vascular endothelial growth factor, and bone morphogenetic proteins. It is well known that these factors play a key role in the process of regeneration of damaged tissues. The first stem cells which were widely described in literature are the cells isolated from bone marrow (Bone Marrow Stromal Cells – BMSCs) (Zuk et al., 2002). They are considered to be the progenitor cells of osteoblasts, chondroblasts and adipocytes. They are characterized by the presence of the following surface antigens CD73, CD90, CD105, and they do not express hematopoietic antigens (Zuk et al., 2002). Most frequently, especially in the case of large animals, the cells are isolated from the iliac crest or the sternum. They constitute only from 0.01 to 0.0001% of mononuclear cells of bone marrow and their number is closely correlated with the patient's age (Gonzales et al., 2009; Lai et al., 2011). In addition, the procedure of collecting bone marrow carries a high risk of infection every time, and in the case of collecting it from the sternum the further risk of damaging pericardial sac exists. Importantly, a small number of BMSCs cells collected from bone marrow requires a comparatively long in vitro culture, which translates to the need for delaying the therapeutic injection. For these reasons it becomes natural to search for other, more effective from cyto-physiological and practical viewpoint, sources of stem cells. Such a population constitutes stem cells isolated from the

adipose tissue (ASCs). These cells display expression of similar cell surface antigens to BMSCs and their collection procedure is undoubtedly safer and does not require general sedation of the patient. ASCs cells in contrast to BMSCs do not have antigen CD106 but they express the marker CD49d (Minguell et al., 2001; Strem et al., 2005; Zhu et al., 2008). Routinely, the adipose tissue for therapeutic purposes is collected under local anesthesia from the hypodermis in the case of small animals, and from around the base of the tail in the case of large animals. ASCs cells in in vitro conditions, similarly to BMSCs cells, differentiate into bone and cartilage cell tissues, which offers the possibility of their wide application in a clinical setting, as in the treatment of diseases of the loco-motor system. These diseases in the case of dogs and horses are under intense research aimed at developing the most effective methods and forms of treatment. Diseases and dysfunctions of the musculoskeletal system are disorders which have genetic origins or result from incorrect use in sports, injuries, improper nutrition or are related to overweight of the animal. The result is pain, reduced mobility, and consequently a significant reduction of life quality. Orthopedic diseases include congenital, developmental, traumatic and degenerative disorders. The most common musculoskeletal disorder in dogs is osteoarthrosis. It is a chronic, multi-etiological dysfunction syndrome wherein as a result of biological and mechanical factors there occurs imbalance between the process of destruction and regeneration of the joint cartilage and the subchondral bone layer. Characteristic of this disease is a secondary inflammatory process of the synovial membrane of the joint, which is why the disease is commonly known as Osteoarthritis – OA. In the course of the disease there occurs gradual thickening and loss of elasticity of the ligaments and joint capsule, resulting in a limitation of joint mobility and progressive muscular atrophy (Tuglu et al., 2010; Sandell, 2012). The degeneration process is accompanied by gradually increasing effort pain and stiffness of walk after rest, lasting no longer than 20 to 30 minutes and relieving on movement. The radiographic picture of joints initially shows a slight narrowing of the joint space and sclerosis of subchondral bone layer. Over time osteophytes appear at the edges of the joint surface and the footprint of the bone epiphysis is distorted. The synovial fluid cytology shows characteristic increase in the number of mononuclear cells (over 3000/μl) with a high proportion of synoviocytes. In advanced cases large numbers of erythrocytes and erythrophages are observed, which is a sign of severe damage to the synovial membrane.

In the case of horses one of the best described orthopedic disorders is bone spavin (Egenvall et al., 2010). Impaired movement is observed in the initial stage of the disease. After a longer rest the horse's walk is stiff, which discontinues after a few minutes of movement. The advanced stage of the movement disorder is manifested by significant lameness, with deformations observed on the anterio-medial side of the tarsal joint. The disease is most common in older horses aged 8−10 years, but can also occur in young animals as so called "juvenile spavin". The anatomy of the joint is considered the contributory factor to spavin occurrence. Greater force is applied to the central tarsal bone and tarsal bones I and II on the medial side. Other factors leading to the development of spavin include wrong posture (convergent / off line posture and its combinations), faulty shoeing, moving on hard surfaces. The spavin disease

may start to develop already during the first intensive training runs performed to achieve high physical performance in a short time. The greater overloads, e.g. due to jumps, the greater macro and micro injuries of the cartilaginous tissue, which have been considered the most important etiologic factor contributing to spavin disorder. Another cause of spavin is abnormal metabolism and inadequate nutrition, including disturbances in vitamin balance.

In the cases of severe lameness in both dogs and horses, surgery is the treatment of choice. In less severe cases, conservative drug therapy is applied using non-steroidal or steroidal anti-inflammatory drugs (Holloway et al., 2012). Full recovery is difficult due to the formation of the repairing deficient scar tissue and insufficient degree of tissue regeneration. Because the treatment with anti-inflammatory drugs only reduces the symptoms that accompany the degeneration process, an attempt has been made to apply a novel method using auto-logic stem cells. It has been proven that these cells release a number of factors desirable for regeneration such as: aggrecan, connective tissue growth factor (CTGF), bone morphogenetic proteins (BMPs), transforming growth factor (TGF) and others.

Purpose. The aim of study was the use of cells called mesenchymal stem cells (MSC) as an alternative treatment method of osteoarthritis. Stem cells are defined as a self-renewing population capable of differentiating into many distinct types. However, the division was adopted determining both their source and their capabilities. Embryonic stem cells (ES) are the most primitive form of cells. They form the embryo, but also placenta, giving rise to all the tissues of the developing organism – they are totipotent. They are characterized by unlimited ability to divide. The second subgroup are the mature stem cells found in individuals in the postnatal life period in many mature tissues. It is believed that they form a reserve of undifferentiated cells necessary for maintaining the structure and the function of a given tissue. The possibilities of their differentiation are usually limited to a form of tissue within a given germ layer (they are pluripotent), however exceptions to the rule have been shown experimentally. In vitro studies have repeatedly demonstrated their high capacity to proliferate over a relatively long period of time, in addition to the ability to transform into a fully organized tissue fragments (Hackett, 2013). They are convenient research material because their isolation does not entail ethical controversy as in the case of embryonic stem cells. Publications report new types of tissue which showed the presence of mature stem cells (Minguelle et al., 2001; Zuk et al., 2002; Zhu et al., 2008; Gonzales et al., 2009; Lai et al., 2011). Their traditional and earliest known sources are bone marrow and umbilical blood. It is currently believed that these cells are found in every tissue but in different numbers. They were found e.g. in the skin, epithelia or even the brain (Tuglu et al., 2010). There are more of them identified in tissues undergoing constant self-regeneration. These cells were first isolated in 2001 from the adipose tissue obtained by liposuction. They have been proven to be multipotent by transforming them in vitro into several types of tissues. Despite differences in the expression of certain surface proteins, their nature is similar to those received from the bone marrow. Low invasiveness is the key advantage of this method of obtaining. The number of mesenchymal cells isolated during one process is also higher due

to larger volume of the source material. Cells divide well under culture conditions and maintain their multipotent capacity for many weeks. This approach allows obtaining a multiple of the original explant. Grafts of autologous adipose tissue derived mesenchymal stem cell (AD-MSC) proved to be safe and effective for the patient.

The joint degeneration disease – osteoarthrosis – is a chronic incurable disease of inflammatory and degenerative etymology. It is caused by damage to the cartilaginous layer induced by long-lasting inflammation as a result of imbalanced anabolic and catabolic processes in the cartilage. Even after the elimination of inflammation, the tissue does not regenerate. Exactly these observations led me to develop a new therapeutic method using autologous graft AD-MSC not only to reduce the ongoing inflammatory process but also to initiate regeneration processes. One of the factors secreted by AD-MSC is IL-1ra, which as the receptors antagonist for IL-1 inhibits the cascade of postinflammatory reactions. Furthermore, the growth factors sent to the environment support the reconstruction of cartilage hence enabling it to return to the original physiological condition. A comparably high significance in filling in defects in the cartilage holds the sole differentiation of stem cells towards chondrocytes. The past encouraging results of analyzes carried out by scientific research teams in the world give the basis to more detailed studies of autologous stem cell injections in the course of the loco-motor system related diseases in dogs and horses (Black et al., 2008; Guercio et al., 2012; Marycz et al., 2012; Burk et al., 2013). Experiments with the use of autologous stem cells were performed in dogs (Experiment No. I) and horses (Experiment No. II).

Materials and methods of research

Twelve dogs have been chosen for experiment No. I (7 males and 5 females) aged 5 to 11 years, including: German Shepherd (4), Labrador (2), Boxer (2) and a mixed breed (4). All animals were diagnosed with the degeneration of the elbow joint, lasting for at least 5 months. The body weight of the patient dogs ranged between 25−50 kg of body weight. Before the selection all the patients underwent a blood test to assess their general condition and exclude comorbid disorders. During the clinical examination all dogs exhibited abnormal gait characteristic of osteoarthritis (OA): lameness – both in gait and trot (2nd and 3rd degree in 6 grade scale) and limited mobility as well as pain during manipulation. The computed tomography (CT) which was performed on all the chosen dogs revealed the 2nd and 3rd degree of degeneration of joints (in four-grade scale). Additionally punctures were performed and the collected liquid samples were transferred to the laboratory for test. Due to the similarity of clinical symptoms the dogs were divided into 2 groups: 8 dogs formed the test group and the remaining 4 dogs formed the control group. The dogs from the first group were treated with autologous stem cells. The dogs in the control group received non-steroidal anti-inflammatory drugs (NSAIDs) − mavacoxib in the dose of 2 mg/kg of body weight (Trocoxil) for a period of 6 months. The dogs from the test group were subjected to sedation with medetomidine in a dose of 20μg/kg of body weight, i.m. and butorphanol in a dose of 0.2 mg/kg of body weight i.m. The area of the iliac crest was prepared according to the principles of surgical cleanliness. Subsequently infiltra-

1. Clinical study of dogs with osteoarthritis on the first day of experiment *(n = 12)*

	Procedure	Lameness in gait (1-6)	Lameness in trot (1-6)	Pain during manipulation (1-3)	Scope of movement (1-4)	Degree of disability (1-5)
Patient 1	SC	3	3	3	3	3
Patient 2	SC	2	3	2	2	2
Patient 3	SC	2	2	3	2	3
Patient 4	SC	3	3	3	3	3
Patient 5	SC	2	3	2	2	2
Patient 6	SC	1	2	2	2	2
Patient 7	SC	3	3	3	4	3
Patient 8	SC	2	1	2	2	2
Patient 9	control	3	3	3	3	3
Patient 10	control	2	3	3	3	3
Patient 11	control	3	3	3	3	3
Patient 12	control	3	2	2	3	3

Note: SC – autologic stem cells administered; control – NSAIDs – mevacoxib 2 mg/kg.

tion anaesthesia was administered with 2 % lidocaine solution and the sample of about 5 grams of fat was collected in order to isolate and multiply stem cells in laboratory conditions. The samples of the adipose tissue were processed under sterile conditions using a laminar chamber of the 2nd class of cleanliness. The sample sections were thoroughly rinsed with Hanks Salt solution, crushed and

2. Examination of the synovial fluid collected from the elbow joint in dogs with osteoarthritis on the first day of experiment *(n = 12)*

Patient	Colour	Clarity	Viscosity	Leukocyte number (×103)	Neutrophils (%)	Mononuclear cells
1	colorless	slightly cloudy	reduced	3	9	98
2	light yellow	slightly cloudy	correct	4	10	96
3	colorless	slightly cloudy	correct	3	5	94
4	colorless	transparent	correct	4	12	96
5	colorless	slightly cloudy	reduced	3	14	98
6	light yellow	transparent	correct	5	10	93
7	colorless	slightly cloudy	reduced	5	8	95
8	colorless	transparent	reduced	4	10	94
9	light yellow	transparent	reduced	5	9	90
10	colorless	slightly cloudy	reduced	6	12	92
11	light yellow	slightly cloudy	correct	4	8	94
12	light yellow	transparent	correct	3	7	92

devoided of blood and blood vessels. The samples were then etched with collagenase (5mg/mL) for 40 minutes at 37 °C and then centrifuged for 10 minutes at the force of 1200xg (Yasuda et al., 2011). The unetched tissue was removed together with the supernatant and the mononuclear cells located at the bottom of the tube were suspended in the culture medium (DMEM: F12 / Ham's supplemented with 10 % bovine fetus serum and 1 % penicillin / streptomycin / amphotericin b) and placed in culture flasks T-25cm^3. Such cultures were maintained

3. Examination of synovial fluid sampled from the tarsal joint of the horses diagnosed with spavin on the first day of the experiment *(n = 16)*

	Apperance	Clarity	Viscosity, cm	Total protein, g/dL	Mucin clot	Leukocytes, cells/μL	Neutrophils, %
1. Test group	yellow	opaque	2	2,7	correct	8.000	< 15
2. Test group	yellow	opaque, single flocculent material	< 2	3,5	relatively good	5.500	< 12
3. Test group	yellow	clear	< 2	2,8	correct	5.000	< 10
4. Test group	yellow	clear	4	3.5	correct	4.600	< 15
5. Test group	light yellow	slightly cloudy	< 2	2,6	correct	8.500	< 15
6. Test group	yellow	opaque, single flocculent material	1	2,7	relatively good	7.000	<15
7. Test group	yellow	opaque	< 2	2,9	correct	6.000	< 13
8. Test group	light yellow	clear	4	3,0	correct	5.000	< 15
9. Test group	yellow	flocculent opaque	2	2,4	relatively good	9.000	< 13
10. Test group	yellow	slightly cloudy	2,5	3,2	good	7.500	< 14
11.Comparison group	yellow	clear	2	3	correct	5.000	< 12
12.Comparison group	light yellow	slightly cloudy	< 2	2,5	correct	7.500	< 15
13.Comparison group	light yellow	clear	1	2,8	correct	4.500	< 13
14. Control group	yellow	opaque, single flocculent material	< 1	2,7	relatively good	8.000	< 15
15. Control group	yellow	clear	< 2	2,4	correct	6.000	< 10
16. Control group	light yellow	clear	< 1	2,6	correct	4.000	< 15

Note: Results of the research conducted on dogs (Experiment no. I.).

at 37 °C/5 % CO_2/95 % humidity for 3−4 days. At full confluency, the cells were passed into culture vessels T-75 (using the reagent TrypLE Express) and grown in the proliferation medium (DMEM – 4500 mg/L glucose, 15 % bovine fetus serum) for 9 days. Microbiological purity was controlled during the entire cycle with the use of an inverted microscope. Then the cells were collected, suspended in sterile physiological saline, counted using a hemocytometer and their viability was checked using the trypan blue dye test. One ml of the suspension (5×106 cells/ml) was collected in a syringe and immediately transported to the clinic, where after preparation of the injection area in accordance with the principles of surgical cleanliness it was injected into the elbows of 8 dogs from the test group.

The following tables show the dogs' qualification criteria for Experiment I.

Parallel studies were conducted in horses diagnosed with the spavin disease confirmed by clinical examination, X-ray examination and diagnostic anesthesia (Experiment II). The experiment group consisted of 16 animals aged 8 to 14 years, 10 of which constituted the test group, 3 formed a comparison group, and subsequent 3 formed a control group. The adipose tissue samples in the amount of 3–4 g each were collected from around the base of the tail of the 10 horses under local anesthesia (40 mg Lignocaina/mL). The collected material was placed in sterile saline solution and transferred to the laboratory for propagation of autologic stem cells. The resulting suspension in the amount of 5×10^6 cells in 1ml was deposited into the tarsal joint. The comparison group was administered 1 mL betamethasone intraarticularly, preparation Diprophos (6.43 mg betamethasoni dipropionas + 2.63 mg betamethasoni natrii phosphas) after preparing the injection area in accordance with the principles of surgical cleanliness. The remaining three horses in the control group were only recommended limited movement.

The control study consisted in examination of synovial fluid, which was conducted on the first day and after 90 and 180 days – clinical tests on days 30, 60, 90 and 180, and X-ray, which was performed on day 180 of the experiment. Additionally 6 horses (2 horses in the test group, 2 in the comparison group, and 2 in the control group) underwent scintigraphic examination on days 1, 90 and 180.

The following tables show the selection criteria of horses in Experiment II.

Results of the research and their discussion

Clinical examination on the first day of the experiment showed signs of lameness of the 3rd and 2nd degree in all 12 dogs in some cases manifested more strongly in gait and in other cases in trotting (Table 1). On day 60 a slight improvement was observed in the test group that underwent stem cell therapy. The dogs demonstrated reduced lameness in the gait and trot as well as a lower reaction to pain on manipulation. In contrast, the control group exhibited a slightly higher pain reaction. On day 90 the clinical test results in the control group remained unchanged. However, the results of tests in the test group continued to improve. The prior symptoms such as stiff gait, lameness or pain were observed in only 3 out of 8 dogs. The clinical tests performed on day 180 in the control group showed signs of gait disturbance at the unchanged level. However, higher pain reaction was observed especially on manipulation. The dogs undergoing treatment with the use

4. Examination of synovial fluid sampled from the elbow joint in the dogs with osteoarthritis on day 90 of the experiment *(n = 12)*

Patient	Colour	Clarity	Viscosity, cm	Leukocyte number	Neutrophils, %	Mononuclear cells
1	colourless	clear	4	2	4	95
2	light yellow	slightly cloudy	3	2	3	93
3	colourless	clear	5	1	0	94
4	colourless	clear	3	3	5	93
5	light yellow	slightly cloudy	reduced	4	5	96
6	light yellow	clear	5	2	3	92
7	colourless	clear	4	2	2	93
8	colourless	clear	4	3	3	91
9 control	light yellow	slightly cloudy	3	6	7	93
10 control	colourless	slightly cloudy	reduced	7	6	94
11 control	colourless	slightly cloudy	reduced	6	8	96
12 control	light yellow	slightly cloudy	3	5	10	94

Note: Control – NSAIDs – mevacoxib 2 mg/kg.

of autologic stem cells did not show any movement disorders. Symptoms such as lameness in gait and trot and pain on manipulation have been completely reduced. Only the scope of motion of the joints remained at a stable, limited level.

Synovial fluid analysis results corresponded to the clinical picture of the patients. On the 1st day of the experiment all 12 dogs showed traits characteristic of chronic degeneration (Table 2). The level of leucocytes was below 6×103/L, neutrophils between 5−12 % and mononuclear cells ranged between 86−98 cells per field. The color of the collected synovial fluid ranged from light yellow to colorless, and in 7 cases it was slightly cloudy. On day 90 the parameters for the dogs in the control group deteriorated. The liquid became slightly turbid and a quite noticeable increase was observed in the levels of neutrophils, leukocytes and mononuclear cells. The test results of the treated group improved. The synovial fluid remained cloudy in only 2 cases and reduced viscosity was still observed in only 1 out of 8 dogs. Other parameters remained within normal limits, the number of leucocytes between 1−4×103/L and the neutrophil value dropped below 5 %. On day 180 the analysis of the synovial fluid in the control group showed that the results obtained were similar to those obtained in the examination on day 90. The leukocytes remained at the same level and a slight increase was observed in the number of neutrophils and mononuclear cells. However, the synovial fluid in patients in the test group did not show characteristics of chronic inflammation. The levels of leukocytes, neutrophils and mononuclear cells were normal. In one case persistent turbidity of the

fluid was reported but in the remaining patients it was clear, ranging from light yellow to colorless of proper viscosity.

The CT scan performed on the first day of experiment proved that the image of elbow in all the dogs was characteristic of a chronic degenerative disease. On day 180 the inflammation persisted in dogs in the control group but in those undergoing stem cell therapy only the persistent lack of mobility in the joint was observed. No features characteristic of an ongoing inflammation were observed.

Results of the research on horses (Experiment no. II): In the experiment on horses (II), the clinical test performed on the first day of the experiment exhibited strong lameness in all animals under research. The X-ray images showed slight degenerative changes characteristic of the early manifestations of the spavin disease in the tarsal joint. The scintigraphy performed on the first day on all six horses exhibited a strong concentration of radionuclide in the tarsal joint area called a hot-spot proving an ongoing inflammation. In a clinical trial in 10 horses undergoing stem cell therapy (horses no. 1−10), there was no change in the clinical picture observed on day 30 after implantation; however the degree of lameness decreased significantly after 60 days. In the case of horses of the comparison group which received the steroidal anti-inflammatory drugs (horses no. 11−13), significant improvement and the absence of clinical symptoms were observed after 30 and 60 days of the experiment. In the control group (horses no. 14−16) lameness remained unchanged after 30 and 60 days of the experiment.

5. Examination of synovial fluid taken from the elbow joint in the dogs with osteoarthritis on day 180 of the experiment *(n = 12)*

Patient	Colour	Clarity	Viscosity, cm	Leukocyte number	Neutrophils, %	Mononuclear cells
1	colourless	clear	4	1	3	91
2	light yellow	slightly cloudy	3	3	4	86
3	colourless	clear	5	2	0	92
4	colourless	clear	3	3	4	90
5	light yellow	clear	3	3	4	92
6	light yellow	clear	5	2	3	92
7	colourless	clear	4	3	2	91
8	colourless	clear	4	2	2	90
9 control	light yellow	slightly cloudy	3	7	7	95
10 control	colourless	slightly cloudy	reduced	8	7	98
11 control	light yellow	slightly cloudy	reduced	6	10	98
12 control	light yellow	slightly cloudy	reduced	5	12	97

Note: Control – NSAIDs – mevacoxib 2 mg/kg.

The scintigraphic examination after 90 days showed no symptoms of arthritis in 4 horses (2 of the test group and 2 of the comparison group), while the inflammation still persisted in 2 horses in the control group.

The clinical study after 90 days showed no signs of lameness in 13 horses (10 of the test group and 3 of the control group), while in 3 horses in the control group the signs of lameness were still strongly manifested.

The clinical examination after 180 days identified:

- test group – no lameness
- comparison group – average degree of lameness
- control group – slight decrease in the degree of lameness.

The scintigraphic examination after 180 days showed persistent inflammation in 2 horses of the control group, and a relapse of inflammation in 2 horses of the comparison group, whereas in the case of horses from the test group no concentration of a radiopharmaceutical (technet 99) was observed around the tarsal joint, which proved the lack of inflammation.

The X-ray images after 180 days showed no significant changes compared to the examinations made on the first day of the experiment. The synovial fluid examination on the first day showed symptoms of inflammation in all horses under experiment.

The results of the examination performed after 90 days improved significantly in the test group and the comparison group. The synovial fluid showed no inflammatory features. The appearance, viscosity, total protein concentration and the number of cells were within the norm for a healthy joint, which indicated no significant differences in the initial treatment of both groups. In contrast, the reduction in the number of neutrophils observed in the control group indicated the transition of the inflammation into its chronic form.

Upon receiving similar results of the therapy in the comparison group and the test group it was decided to repeat the examination of the synovial fluid after 180 days. No significant differences compared to the examinations performed after 90 days were noted in the control group. In the case of the test group undergoing stem

Fig. 1. Changes in the amount of neutrophils in synovial fluid in the test group of 10 horses treated with stem cell therapy

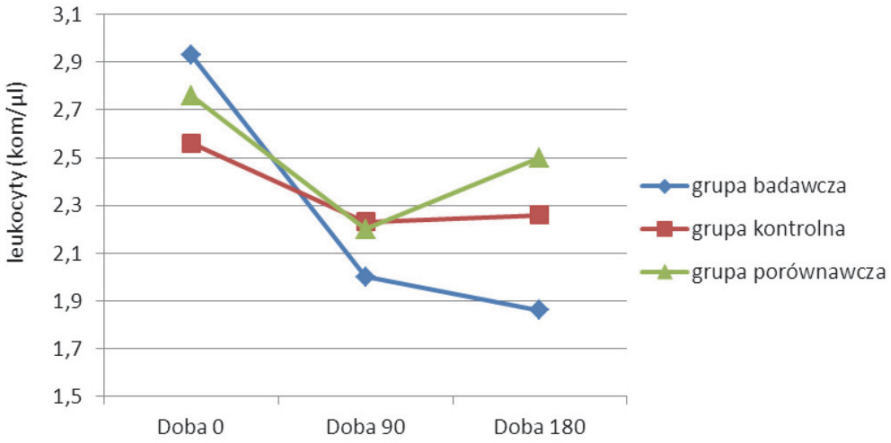

Fig. 2. Change in the amount of leukocytes in the synovial fluid in the three groups under research within 180 days

cell therapy, the results were still within the normal range of a healthy joint, and the level of leukocytes continued to drop (it was impossible to repeat sampling in 2 out of 10 horses). In the case of comparison group treated with steroidal anti-inflammatory drugs the examination performed after 180 days showed a significant increase in the number of inflammatory cells and total protein concentration, a slight decrease in viscosity and in 2 cases (out of 3 under examination) a change of color and the appearance of turbidity in comparison to the examination performed on day 90 of the treatment. The obtained results indicate the reemission of inflammation and short-term effects of treatment with anti-inflammatory drugs.

To sum up, the treatment with autologic stem cells showed a significant improvement in both dogs and horses under research. The symptoms such as stiff gait, pain and lameness were significantly reduced while in the control group, which did not undergo any treatment, they significantly increased. The novel method of treatment using an intraarticular injection of autologic stem cells meets modern requirements for therapies. It is a safe method which provides long-term anti-inflammatory effect, improves joint mechanics and does not cause harmful side effects.

In the case of degeneration of the elbow joint in dogs or spavin disease in horses, the autologic stem cells therapy can reduce discomfort and diminish clinical symptoms associated with these diseases, which has a significant impact on improving the functioning of the animal, and especially on inhibiting the progression of the disease and the elimination of the constant application of analgesics.

In view of the fact that only a slight reduction of pain symptoms was observed in the initial phase after the implantation of stem cells in both dogs and horses, it was decided to further investigate the effect of selected steroid and non-steroidal anti-inflammatory drugs in in vitro conditions on the morphology, ultrastructure and cytophysiological activity of stem cells. The investigated drugs included steroids (betamethasone / methylprednisolone acetate) routinely used in clinical setting as well as nonsteroidal (metamizol). The results indi-

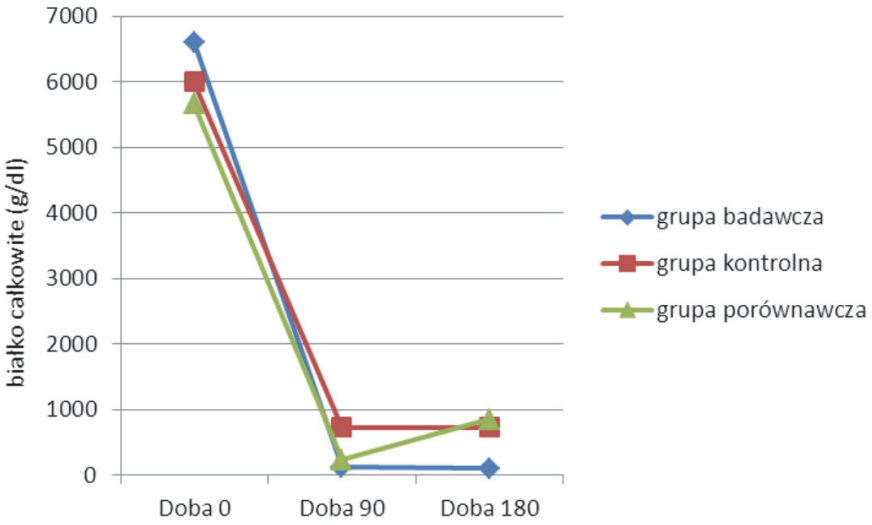

Fig. 3. Change of the total protein concentration in the synovial fluid in 16 horses under research within 180 days

cate a positive cellular response of ASCs to both tested drugs depending on the dosage used. The stem cells isolated from the adipose tissue of horses (equine adipose derived mesenchymal stem cells – EqASC), were characterized by a higher rate of proliferation when the applied dose amounted to 0.01 mg/mL of both examined drugs. In addition, the discussed dose brought no morphological and ultrastructural changes in the cultured cells. The cells preserved fibroblast-like shape, bipolar morphotype, and centrally arranged nuclei. The same concentration of betametzone in turn significantly inhibited cell divisions prolonging population doubling (Population Doubling Time – PDT) of the ASCs. The culture of EqASC (Equine adipose derived mesenchymal stem cells) and CaASC (Canine adipose derived mesenchymal stem cells) using methylprednisolone acetate at a concentration of 0.01 mg/mL and 0.1 mg/mL resulted in an increase in the rate of proliferation of both studied stem cell populations. The 1 mg/mL concentration of methylprednisolone acetate and betamethasone completely inhibited cell division in CaASC EqASC. Stem cells isolated from the adipose tissues of dogs and horses, cultured in the presence of both drugs at concentrations of 0.01 and 0.1 mg/mL, showed no morphological changes in the course of seven days of the experiment. The cells were characterized by a normal morphotype and created numerous intercellular junctions. The addition of 1 mg/mL of each of the test drugs to EqASC and CaASCs cultures led to significant changes in the cell morphology. Numerous apoptotic and necrotic cells were observed in the culture. Both drugs tested at doses of 0.1 mg/mL and 0.01 mg/mL stimulated EqASC and CaASC cells for the synthesis and secretion of membrane microfragments (MVs), which correlates with the high cytophysiological activity of the tested cells. The results indicate the possibility of incubating the stem cells in the pre-transplantation phase in order to increase their secretive capability and thus enhance their regeneration abilities (Ratajczak et al., 2013).

Similar results were obtained in the culture of EqASC and CaASC stem cells with nonsteroidal drug – metamizol. The application of the lowest concentration, i.e. 0.01 mg/mL, significantly stimulated the cells' division, thus shortening the population doubling time. The cells in the culture preserved the fibroblast-like morphotype with centrally located nuclei. In addition an increased number of intercellular junctions was observed together with increased cytophysiological activity of cells which was manifested by the presence of numerous membrane microfragments (MVs) located on the surface of the tested cells. The lowest concentration of the test drug resulted in an intensive synthesis and secretion of MVs in the case of CaASC, while in the case EqASC only single membrane microfragments were observed. The application of higher doses of the test drug, i.e. 0.01 and 0.1 mg/mL, inhibited the proliferation of the examined cells and consequently resulted in a complete inhibition of growth particularly when the dose of 1 mg/mL was applied. In the case of 0.1 mg/mL dose both cell populations exhibited uniform distribution and preserved their proper fibroblast-like shape. In the case of the highest concentration of the test drug, numerous apoptotic bodies were observed in both EqASCs and CaASCs cultures.

Conclusions and future perspectives of the study

The obtained results allow drawing the conclusion that the combination of the lowest concentrations of metamizol may significantly increase the cytophysiological activity of the stem cells accompanied by the lack of morphological changes. The obtained data can be a valuable source of information for practicing veterinary doctors who introduce stem cells to treat musculoskeletal system diseases in both small and large animals.

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Якоб Нікпон, Поліна Зелінська (2020). АУТОЛОГІЧНА ТЕРАПІЯ СТОВБУРОВИМИ КЛІТИНАМИ У СОБАК І КОНЕЙ З ХРОНІЧНИМ ОСТЕОАРТРИТОМ.

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Анотація. *Однією з найбільш швидко прогресуючих галузей ветеринарної медицини за останнє десятиліття була і залишається регенеративна медицина. Основна увага приділяється реконструкції пошкоджених тканин / органів для відновлення їх фізіологічних та функціональних показників. Метою даного дослідження було використання мезенхімальних стовбурових клітин (МСК) як альтернативного методу лікування остеоартриту. Для експерименту було обрано 12 собак у віці від 5 до 11 років. У всіх тварин діагностували дегенеративні зміни у ліктьовому суглобі. Собак розділили на 2 групи: 8 собак сформували тестову групу, а решта 4 собаки – контрольну групу.*

У собак першої групи були застосовані аутологічні стовбурові клітини. Собаки контрольної групи отримували нестероїдні протизапальні препарати (НПЗП) – мавакоксиб протягом 6 місяців. Клінічне обстеження в перший день експерименту показало ознаки кульгавості 3-го та 2-го ступеня у всіх 12 собак. Клінічні тести, проведені 180 дня у контрольній групі, показали ознаки порушення ходи на незмінному рівні. У собак, які пройшли лікування із застосуванням аутологічних стовбурових клітин, не виявлено жодних порушень руху. Отримані результати дозволяють зробити висновок, що поєднання найнижчих концентрацій метамізолу може значно підвищити цитофізіологічну активність стовбурових клітин, що супроводжується відсутністю морфологічних змін. Отримані дані можуть бути цінним джерелом інформації для практикуючих лікарів-ветеринарів, які впроваджують стовбурові клітини для лікування захворювань опорно-рухового апарату як у малих, так і у великих тварин.

Ключові слова: *стовбурові клітини, мезенхімальні стовбурові клітини (МСК), остеоартрит, нестероїдні протизапальні засоби (НПЗП), мавакоксиб*

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