Journal of Animal and Veterinary Advances 11 (23): 4324-4331, 2012
ISSN: 1680-5593

The Therapeutic Effect of Autogenic Adipose Derived Stem Cells Combined with Autogenic Platelet Rich Plasma in Tendons Disorders in Horses *in vitro* and *in vivo* Research

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Abstract: Naturally, occurring tendons injuries including superficial digital flexor tendinopathy are the most frequent musculoskeletal disorders in performance horses. Conventional methods of treatment with non steroidal and steroidal anti-inflammatory medicaments in majority of cases lead to scar formation, reducing the quality and efficiency of tissue regeneration. Novel approach is aimed to use cells naturally present in an organism as regeneration enhancing factor. In conducted research, the intralesional injections of autologous adipose derived stem cells combined with autologous platelet concentrate therapeutic potential was investigated in horses with 8-12 weeks duration superficial digital flexor injury with severe scaring. Collected by clinical examinations data showed positive effects of autologous, adipose-derived mesenchymal stem cells combined with autologous platelet rich plasma injections on regeneration processes in the course of superficial flexor tendon injures in horses. On the basis of ultrasound examination, it was proved that the quality of healed tissue was significantly higher in experimental group, comparing to control group. Obtained results confirmed the beneficial pro-regeneration effects of stem cells/platelet concentrate combined injections. The obtained data may also serve as valuable source of information about morphology and behaviour of fat stem cells in culture or platelets appearance.

Key words: Tendons, novel, tissue regeneration, adipose-derived mesenchymal stem, ultrasound

INTRODUCTION

Equine Musculoskeletal System biomechanics which has evolved to extremely sophisticated system, allows high speed and high efficiency of the horse. Concurrently, horses become more susceptible to both-acute and chronic injuries, connected with intense motion. Any injury to equine tendons or ligaments is a serious threat to his short-term soundness and his future career prospects (Hill *et al*., 1986). Tendon and ligament strains and sprains are very common injuries in performance horses. Disease entities connected with excessive sport exploitation are among others, tears or ruptures of Superficial Digital Flexor Tendon (SDFT) and Deep Digital Flexor Tendon (DDFT) structures that are burdened mechanically the most in horses subjected to training (Goodship, 1993). They are characterized by high rate of re-occurrence (about 80%) and difficulties in terms of treatment. Mature tendons lost the ability to adapt the pressures and stresses they are exposed to. As a result of rigorous and improper training, connected with their continual use at close to limit of resilience, they usually undergo progressive degeneration, resulting in tearing and failure of the tendon. Especially in sport horses, damages and inflammatory states of tendons and ligaments system are caused by repeated microinjuries and in a consequence, exceedance of physiological tissue strength (Marr *et al*., 1993). Regeneration of tissues in such kind of injuries is difficult due to ongoing activity of mechanical forces and by limited possibilities of limb immobilization. Traditional methods of treatment include in general, non steroidal or steroidal injections combined with motion restriction. Unfortunately as a result of this kind of therapy usually scar tissue is formed which doesn't allow for restoring full motoric efficiency of affected structures. The possibilities of regenerative

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medicine are thought to be an effective alternative (Crass *et al*., 1992). Procedure being in currently usage, applied especially in cases of musculoskeletal system advanced degenerative changes is mesenchymal stem cells injections. It was proved that they cause a stimulation of reconstructive properties of the injured tissues (Shen *et al*., 2009, 2010). Bone marrow is considered and well recognized source of mature stem cells. However, recently adipose tissue seems to also be a valuable origin of mature stem cells. The stem cells are defined as self-regenerating population of cells which may differentiate in many separate types (Muller *et al*., 2008). Embryonic stem cells are theoretically characterized by unlimited ability to proliferation and after removing of large part of an embryo are able to reconstruct missing fragments. On the other hand mature stem cells including Adipose-Derived Mesenchymal Stem Cells (AD-MSC) and Bone Marrow-derived Stem Cells (BM-MSC) which are present in individuals in postnatal period of life in numerous mature tissues (Ratajczak *et al*., 2008). It is believed that they are a reserve of undifferentiated cells essential to maintain the structure and function of a given tissue. Injections of autologous MSC into damaged tendons seem to give promising results (Smith and Webbon, 2005). The additional opportunity offered by regenerative medicine is application of Platelet Reach Plasma (PRP). Platelets are known as rich source of different tissue growth factors such as: Connective Tissue Growth Factor (CTGF), Vascular Endothelial Growth Factors (VEGF), Transforming Growth Factor (TGF) and others (Marx *et al*., 1998; Marx, 2004; Grainger *et al*., 1995) which are stored in secretory granules and release after activation. They amplify the natural regenerative potential of the injured tendons. In this study, detailed evaluations was conducted on an application of equine autogenic mesenchymal stem cells isolated from fat tissue, combined with autologous PRP as a treatment method of tendons injuries. Before injections the combination of AD-MSC and PRP were precisely examined *in vitro* in order to evaluate the optimal morphological and biochemical properties of therapeutic formula. After detailed *in vitro* analysis AD-MSC/PRP in a form of suspension were applied into tendon core lesion to examine the regeneration effected of this kind of biologically active preparation. Results showed much improvement of healing processes especially in the quality of regenerated tissue formation which showed normal tissue's appearance.

MATERIALS AND METHODS

Experimental therapy course was approved by the Second Local Ethical Comitee for Animal Experiments, localized in Wroclaw, University of Environmental and Life Sciences ul. Chelmonskiego 38C, decision number 177/2010 from 11.15.2010. Research material consisted of 24 horses of various breeds aged between 8-10 years both sexes used in competitive jumping sport with diagnosed 10-12 weeks lasting injury of superficial flexor tendon injury. The severity of tissue destruction was comparable in all investigated horses and was consisted with serious scar tissue formation. Animals underwent trough clinical examination including evaluation of horse's gait, palpation and manipulation (checking for changes in tendon tension and texture and instability of the associated joint). Moreover, diagnostic imaging-ultrasonography were performed. Horses encountered into the study exhibited light lameness. On palpation affected extremity showed the signs of swelling and tenderness over injured tendon. Upon ultrasonographic examinations all horses enrolled into the study revealed hypoechogenic core lesion within digital superficial flexor tendon with associated hyperechogenic areas consisted with scar tissue.

Adipose tissue collection: White adipose tissue was collected from 12 horses from experimental group in amount about 5 g from the tail base area. The material was collected maintaining aseptic conditions under local anesthesia (2% lignocaine). The remaining 12 horses qualified for the experiment serve as a control. Directly after collection, the biopsy material was placed in sterile Hanks' salt (HBSS, Sigma) and immediately transported to the stem cells laboratory (Environmental and Life Science University Wroclaw).

AD-MSC isolation and culture: Cells isolation included fine mincing by surgical scissors, washing in HBSS, clearing from any visible blood traces and vessels; finally enzyme digestion was performed (Zhu *et al*., 2008). Digestion buffer comprised with 0.2% collagenase type I (Sigma) dissolved in HBSS in which shredded tissue was incubated for 30 min in 37°C, 5% CO_2 incubator. After that mixture was centrifuged in Falcon tube 1200×g/10 min (IEC CL31R, Thermo Scientific) which separated solution into three layers with nucleated cells located in the lowest one. Supernatant was discarded, cell fraction was carefully collected and transferred to 25 ccm T-flask with medium (DMEM + 10% FBS + antibiotic solution, Sigma). Cells were maintained in Humidified Incubator (FDHI, Thermo Scientific) in 37°C, 5% CO_2 7 days. Just before reaching confluency by cells, they were washed twice in HBSS, trypsinised (0.2% trypsin, Sigma) and incubated till the majority of them had detached from surface. Trypsin solution with suspended cells was collected, neutralized by complete medium addition, transferred into Falcon tube and centrifuged at 300 g for 5 min. Supernatant was

removed; cell pellet was resuspended in complete medium and counted for total and alive cell yield with Thoma counting chamber by trypan blue (0,4%, Sigma) staining. Afterwards, cells were split to desired density (5×10^4 cells mL^{-1}) and seeded in new T-flasks with 15% of FBS in medium. Twenty passages were maintained. Population doubling times were defined to choose the best grow stage for application. Briefly, cells were counted every day with equation:

$$T_d = \frac{T \times \log 2}{\log (N_t/N_0)}$$

Where:

T = The time of the logarithmic phase of the growth
N_0 = The number of seeded cells
N_t = The number of cells at the end of logarithmic growth phase (Zhu *et al*., 2008)

Microscopic observations and measurements were made daily and of each passage of culture by means of inverted contrast-phase microscope (AxioObserver A1, Zeiss). Additionally, immunofluorescence stainings were performed to check existence of MSC marker panel (CD29, CD44, CD45, CD90 and CD105, Abcam). Cultured cells from passage 0 to passage 12 were fixed with 2.5% paraformaldehyde and incubated with primary antibodies (1:500) for an hour. After that they were incubated again with secondary, fluorochrome conjugated antibodies (goat anti rabbit IgG 488, goat anti mouse IgG 564) also for an hour. After extensive washing, cultures were observed by means of fluorescence inverted microscope and photographed. The obtained populations of stem cells were examined with respect to ability for differentiation towards adipocytes and osteocytes using, respectively: dexamethasone, IBMX, indometacin, insulin for adipocytes; β-glycerophosphate, ascorbic acid, dexamethasone for osteocytes (Zuk *et al*., 2001). Verification of the progress of specific cells differentiation was conducted using special staining (oil red O to visualise lipids, alizarin red staining calcium, respectively). All test conducted aimed at verification if cells cultured fulfil criteria established by International Society for Cellular Therapy (Dominici *et al*., 2006).

Blood collectionl: About 250 mL of full peripheral blood was collected from all experimental horses to the containers with anticoagulant (citrate dextrose, Ravimed). The blood collection was performed 1 day before clinical application, filled containers were then stored in dark place in room temperature.

Preparation of Platelet Rich Plasma (PRP): Full peripheral blood from horses was subjected to the process of double centrifugation according to methods previously described (De Rossi *et al*., 2009) in order to prepare the portion of 10 mL of PRP for 12 horses qualified to the group cured using AD-MSC/PRP combination. Briefly, blood samples were aseptically transferred to centrifuge tubes and centrifuged for 10 min with 300×g. After that the layers with plasma and platelets were transferred to new tubes and centrifuged again with 640×g for 10 min. Finally, the top layers of plasma were discarded with remaining platelet pellets and some plasma. The pellets were then resuspended in remaining plasma and so prepared mixtures were loaded into syringes. Platelet concentrations in whole blood of patients and in PRP destinated to application were checked in Thoma counting chamber with methylene blue staining and compared. Small samples of obtained PRPs was mixed with AD-MSC and prepared to SEM observation. Also samples of PRP without AD-MSC were included to SEM analysis.

Clinical application of elaborated therapeutic mixture (AD-MSC/PRP): The experimental protocol was approved by the Local Committee of Ethics and Animal Welfare of the Environmental and Life Science University Wroclaw. Before applications, AD-MSC/PRP preparations were microbiologically tested. Horses from experimental group received PRP immediately followed with AD-MSC into injured tendon region in several points with total volume of 3 and 1.5 mL (6×10^6 cells), respectively. Every injection was preceded by thorough skin disinfection with ultrasound guidance. Intra-lesional injections were performed under premedication. Anti-inflammatory drugs was avoided during the study in experimental group, except the 1st week after preparation's application if animal was suffering the high pain level (phenylbutazone 1 g/500 kg b.w.). Directly after applications, the animals were excluded from intensive, physical activities for at least 2 weeks and then the proper rehabilitation program was applied. After therapeutic formula applications the injured place was wrapped with bandage containing zink oxidate. Return to full training was planned within 1 year time. The horses from control group were treated with anti-inflammatory medicaments (acetylsalicylic acid 4.5 g/500 kg BW; phenylbutazone 1 g/500 kg BW; local treatment with heparin/acetylsalicylic acid gel) combined with physical activity restriction for 2-3 weeks.

Clinical revision: The quality of healing was evaluated clinically and ultrasonographically. The revisions were conducted at 7th week after injection of selected preparation and at 18th week. In control groups, the rechecks were performed at analogous points of time. The follow up of all horses is still continued at present.

RESULTS

Cell culture: In primary cultures, Stromal Vascular Fraction cells (SVF), namely MSCs, preadipocytes, erythrocytes, smooth muscle and endothelial cells were noticed (Fig. 1).

Mesenchymal cells proliferate in 10%FBS environment so they colonized fully available surface in about 7-10 days (Fig. 2). During first passage about 3×10^6 living cells were detached and split to new flasks. Trypan blue staining confirmed almost 97% vitality. In secondary culture cells were close to rounded of gold-brown colour when examined in inverted contrast-phase microscope. First day after passage, morphology of cells changed, they were more flattened and create relatively long cytoplasmic connections with each other. Next day, cells' morphology was uniform and they were spindle shaped again. Scanning Electron Microscopy (SEM) and immunofluorescence techniques allowed visualizing precisely the ultrastructure of cultured cells (Fig. 3). Not only nuclei with prominent nucleoli but also mitochondria surrounding nucleus, intercellular connections and secretory granules within individual cells were observed.

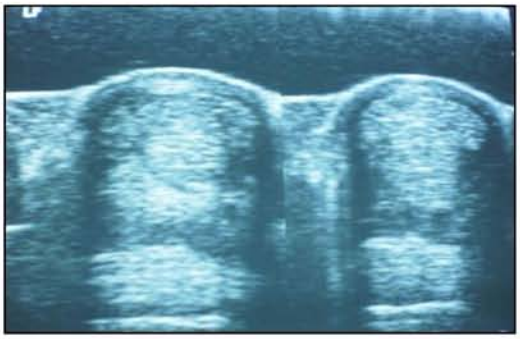

Fig. 1: Primary culture of AD-MSC. Visible other cell types, mainly erythrocytes. Contrast-phase microscope, mag. 100x

Fig. 2: AD-MSC in primary culture expanding surface. Contrast-phase microscope, mag. 100x

Cell's nuclei were comparable including the nucleoli numbers. Precise observations showed movement of endosomal vesicles in cell's cytoplasm. They were mostly found in the perinuclear compartment and just before cell's division they migrate toward to opposite margins. In non-dividing cells (being not during mitosis), they were intensively moving within cell. After 19-20 passages, examined cells were less adhesive more flattened and they were taking up more place. Population doubling times differed from 36 h in passage 3-96 h in passage 16 and higher. Differentiation assays resulted in appearing of lipid droplets and calcium minerals showed on Fig. 4 and 5. Immunofluorescence staining showed positive reactions of all MSC-markers antibodies (Fig. 6).

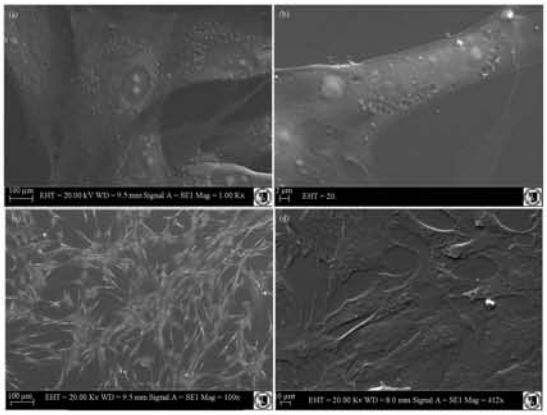

Fig. 3: Scanning electron micrographs; a) appearance of nucleus and perinuclear area of stem cells. Visible prominent nucleoli and mitochondria, mag. 1000x; b) intracellular endosomes relasing its contents, mag. 2000x; c) Structure and growth pattern of AD-MSC in secondary culture, mag. 100x; d) Appearance of stem cells after 19th passages with distinctly flattened morphology, mag. 412x

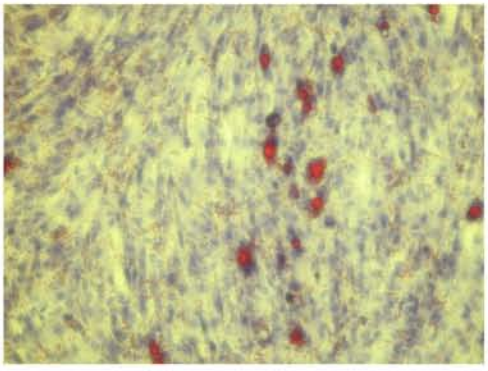

Fig. 4: AD-MSC differentiated into adipogenic lineage. Lipid droplets visualized by oil red o staining, mag. 200x

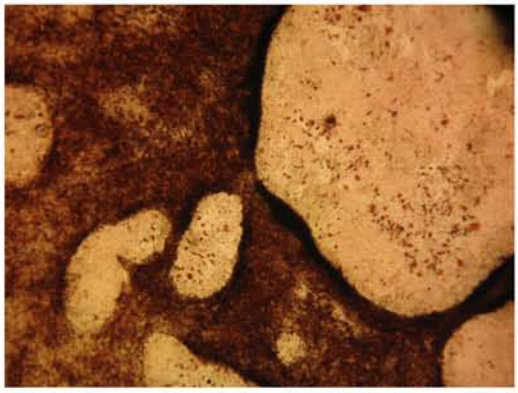

Fig. 5: AD-MSC differentiated into osteogenic lineage. Visible characteristic growth pattern and calcium deposits (red) visualized by alizarin red staining, mag. 50x

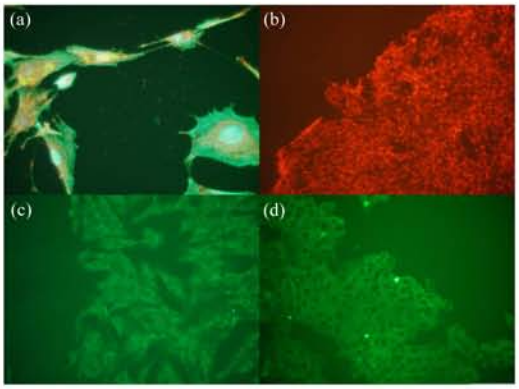

Fig. 6: Immunofluorescence staining of equine stem cells. a) Morphology of cultured cells with visible actin chains (green), mitochondria (orange) and nucleuses (blue), mag. 400x; b) CD29 antigen (red), mag. 100x; c) CD44 antigen (green), mag. 200x; d) CD105 antigen (green), mag. 200x

Expression of CD90 was less pronounced but still visible. No reaction with CD45 antibody was noticed with regards to isotype control. Additionally, the morphology of AD-MSC and platelets together was evaluated by means of scanning electron microscopy. Samples of PRP without AD-MSC addition exhibited mainly the morphology of spherical cells with tiny pseudopodia formation (Fig. 7). Moreover, in some areas the platelets aggregates were also observed. With regards to platelets/AD-MSC mixture the trombocytes exhibited more diverse morphology, namely they had a form of spherical cell with pseudopodia, spread dendritic cells and there were also some spread cells noticed (Fig. 8).

Clinical revision: All horses from experimental and control group at first revision (7th week) after applications

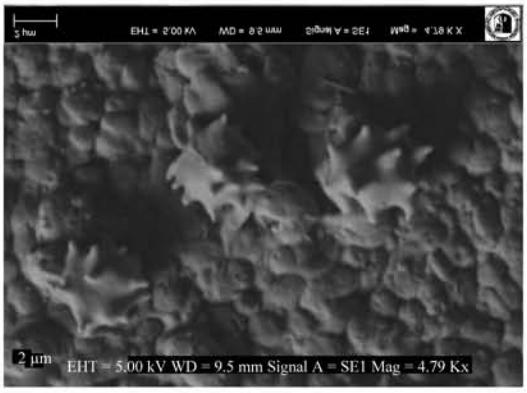

Fig. 7: Inactive platelets pellet's scanning electron micrograph, mag. 4790x

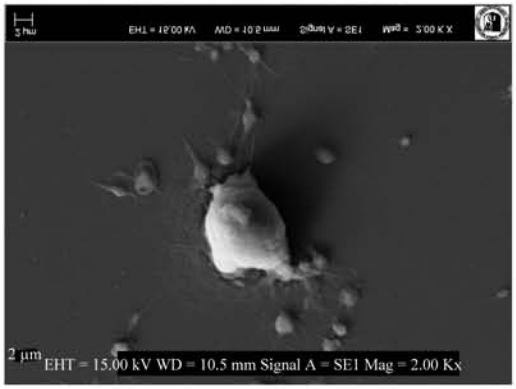

Fig. 8: Scanning electron micrograph of activated platelets with visible elongations in a company of single AD-MSC, mag. 2000x

showed no clinical improvement with regards to severity of lameness. The only improvement noticed at this point was marked reduction of swelling and tenderness of the affected area. No systemic or local adverse tissue reactions were noticed in horses from experimental group. Ultrasound examinations also did not reveal significant improvement, namely hyperechogenic areas consisted with scar tissue formation were still present. In 18th week, revision showed lameness absence in all experimental horses and prominent lameness reduction in control group. Ultrasound examination of horses from experimental group indicated complete resolution of the core lesions with no evidence of prior tendon injuries were found without findings, consisted with scar connective tissue (Fig. 9). In horses from control group, despite clinical improvement, thickening of injured tendon region was noticed on palpation, particularly comparing to opposite leg. Ultrasound examination showed the existence of hyperechogenic areas within superficial digital flexor tendons. Moreover, in some horses there were also hypoechogenic regions, consisted with

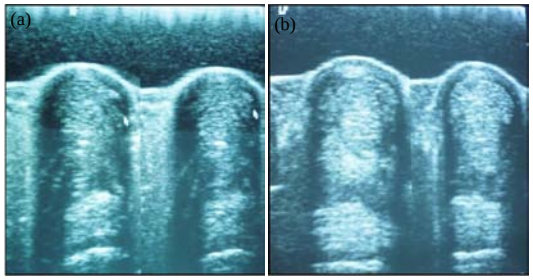

Fig. 9: a) Ultrasonographic pictures of injured tissue site before therapy and after (b:18th week control) with visible regenerated tissue

non-healed hematoma. With regards to control horses despite observed at last revision lameness reduction, ultrasound analysis revealed mark hyperechogenic areas corresponding with persistent scar formations.

DISCUSSION

Diseases of locomotive system in horses are related mainly to wrongly matched training, impaired nutrition, improper hoof correction and individual predispositions (Goodship, 1993). The clinical effect of this disorders is mainly pain, restriction of mobility and in a consequence, often considerable deterioration of life quality and sport efficiency of the animal. In equine medicine, the traumatic factors generated often by extreme sport performance are of the main significance. Disease entities connected to excessive sport exploitation are among others tears or ruptures of Superficial Digital Flexor Tendon (SDFT) and Deep Digital Flexor Tendon (DDFT). In this study, the applicability of bioactive preparations containing AD-MSC and PRP as a therapeutic formula was analysed in horses suffering with superficial digital flexor tendon injuries. Simultaneous application of stem cell and platelets guarantee three main advantages comparing to stem cell alone: providence of tissue growth factors, creation of natural biological scaffold and high stem cells proliferative activity. It should be underlined that the concentration of platelet growth factors (mainly fibroblast growth factor and transforming growth factor) markedly decreases within 2 h after trombocytes activation. For this reason, it was chosen to apply independently ADMSC and PRP directly into core lesion, immediately followed by stem cells injection. Moreover, morphological findings obtained by means of scanning electron microscopy indicated that platelets in PRP without AD-MSC addition exhibited morphology of less activated cells comparing to platelets mixed with stem cells which further confirms the advisability of separate PRP and AD-MSC application (Kawasaki *et al*., 2004; Okroji *et al*., 2009). Both PRP and stem cells are equally important as far as tendon injuries in horses are concern. Many studies have underlined that growth factors released from platelets are involved in tendon healing and can modulate the different cellular processes that lead to the functional recovery of fibers (Molloy *et al*., 2003). Much of the research conducted on adult stem cells has focused on mesenchymal stem cells found within the bone marrow stroma. However, recently also fat tissue seems to be putative source of mature stem cells. There are some differences between AD-MSC and BM-MSC with regards to some surface antigens (e.g., CD49d is present on AD-MSC and not on BM-MSC) (Hass *et al*., 2011; De Schauwer *et al*., 2011).

The main advantages of adipose derived stem cells are: non-invasiveness of technique of their collection, relatively big amounts and high proliferative potential. AD-MSC exhibited unique characteristics distinct from those seen in MSCs including differences in CD marker profile and gene expression (Zhu *et al*., 2008). Conducted immunophenotyping in culture confirmed mesenchymal origin of cultured cells. As it was indicated, MSCs posses the ability to differentiate into tendon-like tissue (Del Bue *et al*., 2007). Direct demonstration of the stem cells survival and synthesizing a tendon-like matrix in horse tendon was not yet obtained. The strong demand to obtain tendon tissue promoting MSC populations seems to be important in optimizing this branch of regenerative medicine. At the end of the study, no evidence of prior tendon lesions was obtained in horses with pre-existing massive scar tissue which suggest the significant rebuilding potential of injected preparation. Delay of beneficial effects for 1st 8 weeks is not easy to explain. It could be explained by diverse interactions between MSCs and extracellular matrix upon different cytokine microenvironment (Kollar *et al*., 2009; Tondreau *et al*., 2009; De Becker *et al*., 2007). It should be underline that not only mixed character of applied formula improve the effectiveness of therapy but also autologous form of AD-MSCs and PRP used in this research exclude graft versus host reactions as well as reduced inflammation to minimum.

CONCLUSION

Collected data showed positive effect of AD-MSC combined with PRP on regeneration processes in the

course of superficial flexor tendon injures in horses. The study showed significant improvement in post treatment evaluation times for lameness, pain and range of motion both in experimental and control horses. On the basis of ultrasound examination it was proved that the quality of healed tissue was significantly higher in experimental group comparing to control group. The obtained results may also serve as valuable source of information about morphology and behaviour of AD-MSC in culture.

Encouraging results could be in future also extrapolated into human medicine field. The followed up examination are planned in the future to evaluate the progress of regeneration process.

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