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Original article

Subchondral bone cyst surgical treatment using the application of stem progenitor cells combined with alginate hydrogel in small joints in horses

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Abstract

One of the most common reasons for horse lameness is subchondral bone cysts (SBCs), which are especially evident in young horse athletes. It is believed that SBC development is strongly associated with an individual's bone growth and/or bone microstructure impairment. Current methods of SBC treatment include pharmacological treatment or surgical procedures which may allow the bone within the cyst to rebuild and be restored to properly developed bone tissue. Thus, we propose filling the SBCs with a 3D complex of alginate hydrogel and autologous adipose derived mesenchymal stem cells (ASCs). We have observed at the *in vitro* level, that this hydrogel complex induces osteogenic and chondrogenic differentiation potential through the upregulation of bone morphogenetic protein, osteopontin, collagen type I and aggrecan mRNA levels. Moreover, we detected the creation of a 3D extracellular matrix (EM). To investigate the complex *in vivo,* we chose 8 horses of varying age suffering from SBC, which resulted in lameness, to undergo experimental surgery. We documented the horses' clinical appearance, lameness and radiographic appearance, to determine that there was clinical improvement in 87.75% of the patients (n=7, out of 8 horses) 6 months postoperatively and 100% (n=8, out of 8 horses) a year after surgery. These results are promising for the potential of this procedure to become the standard in SBC treatment.

Key words: subchondral bone cyst, autologous stem cells, minimal invasive surgery

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Introduction

Subchondral bone cysts (SBCs) are one of the prominent causes for lameness in young horses, which as a consequence results in their exclusion from future sport activity. Lameness often occurs in horses under two years of age and this is believed to be associated with bone growth (Santschi 2011). Some authors credit SBCs as a form of osteochondrosis (von Rechenberg and Auer 2006, Fuerst et al. 2007). On the other hand, it is thought that SBC development is caused by microtrauma and overloading of the bone in young, growing horses. SBC occurrence was also described as a result of bone marrow lesions or a fissure. In all cases of subchondral bone cyst formation, the lack of bone tissue, usually circular shaped, is observed on radiographs. SBCs usually contain fibrous connective tissue and synovial-like fluid. The surrounding bone tissue is usually sclerotic (Fuerst et al. 2007, Baxter 2011). Furthermore, high levels of cytokines generally accompany the inflammation inside the cyst, subsequently resulting in cyst enlargement and further joint damage (Santschi 2011). The SBCs were categorized into 3 radiological types: type 1 – changes of less than 10 mm in diameter, type 2 – changes of more than 10 mm in diameter, and type 3 – flat or irregular changes in bone contour (von Rechenberg and Auer 2006, Baxter 2011, McIlwraith 2015). Most of the cyst communicates with the joint space and this is believed to negatively influence the prognosis. These morphological changes are frequently found in the medial femur condyle, but are not limited to this location and can also be seen in places such as the phalangeal bones, navicular bone, metacarpal or metatarsal bones and many others (von Rechenberg and Auer 2006, Baxter 2011, Mettenleiter 2014). Not only can SBCs be found in different bones but they can also be distributed asymmetrically either in unilateral or bilateral joints (von Rechenberg and Auer 2006, Baxter 2011, McIlwraith 2015). Some SBCs can lead to obvious symptoms such as visible lameness, while other SBCs do not have discernable clinical symptoms and can go undetermined. The prognosis in SBC detection in equine patients are generally thought to be unreliable therefore new therapeutical methods are strongly required.

Most recently, mesenchymal stem cells from adipose tissue (ASCs) have been shown to possess unique therapeutic potential due to their multipotent characteristics, their ability to differentiate into multi-lineages, and their anabolic activity (Marycz et al., Ratajczak et al. 2014, Marędziak et al. 2014, 2015, 2016). One of our previous research projects showed positive clinical effects of ASCs in bone fracture, bone spavin and tendon regeneration (Marycz et al. 2012, Nicpoń et al. 2013). As was previously described, alginate hydrogel promotes the viability and differentiation of the osteocyte cell lines. Moreover, alginate hydrogel has been recognized as a highly suitable candidate for constructing an extracellular matrix which could potentially be used in bone remodeling. Thus the combination of both ASCs and 3D hydrogel seems to be a fully reasonable proposition. Therefore, the aim of the present study was to estimate the application of 3D alginate hydrogel in conjunction with mesenchymal stem cells of adipose tissue in SBC treatment. We believe that the encouraging results of our research may result in producing a novel treatment protocol for minimal invasive surgical SBCs treatment.

Materials and Methods

Experimental procedures

All experimental procedures were approved by the II Local Ethics Committee of Environmental and Life Sciences University (Chelmonskiego 38C, 51-630 Wroclaw, Poland; decision No. 84/2012).

Additional instrumentation

The procedure required a basic surgical set, a drilling machine and a 3.2mm or 4.5mm bit depending on the size of the cyst.

Research group

The clinical part of the study was performed in the EQUIVET Equine Hospital in Poland. The research population was retrospectively chosen from orthopedic patients admitted to the hospital and diagnosed with SBC, positioned in the bones creating relatively narrow joints without the possibility of arthroscopical access to the cyst orifice. Age, breed, gender, admission date, type of performance, degree of lameness and clinical outcome were recorded (Table 1, 2). Diagnostic procedures applied to the horses included: clinical and orthopedic examination, local nerve blocks, in the case of SBCs in the radius intra articular (i.a) block, repeated radiographic examination and, in four cases, MRI examination. The reliability and reproducibility of the examinations were ensured by a consistent and experienced team of veterinarians. All horses were treated surgically through the implementation of autologous stem cells on a matrix of sodium alginate gel according to the methods described below.

Although highly recommended the MRI examination was performed prior to surgery in only 50% of all cases (n=4, out of 8 horses) for economic reasons. MRI was used not only as a diagnostic tool for planning the

Table 1. Patient distribution including breed, age, gender, usage, SBC location, lameness grade and outcome.

No	Breed	Age (years)	Sex	Usage	Limb affected	SBC location	Lameness grade prior surgery	Outcome
1	thoroughbred	<1	M	racing	LF	Mc III distal	3/5	in race training
2	thoroughbred	<1	M	racing	LF	Mc III distal	2/5	in race training
3	KWPN	9	M	Show jumping	RF	Mc III distal	3/5	beginning training after pasture rest
4	warmblood	5	M	Show jumping	RH	P1 distal	3/5	back in training
5	warmblood	7	F	leisure	LH	Mt III distal	3/5	working under saddle
6	warmblood	10	MC	leisure	LF	P2 distal	3/5	working under saddle
7	thoroughbred	<1	M	racing	LF	P1 distal	3/5	pasture (due to age)
8	warmblood	7	F	dressage	LF	Radius roximal	3/5	working under saddle

Table 2. Lameness evaluation and grading on a 5-point scale, according to AAEP lameness scale.

Grade	Definition
0	Lameness not perceptible under any circumstances
1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances (e.g. under saddle, circling, inclines, hard surface)
2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances (e.g. weight-carrying, circling, inclines, hard surface)
3	Lameness is consistently observable at a trot under all circumstances
4	Lameness is obvious at a walk
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move

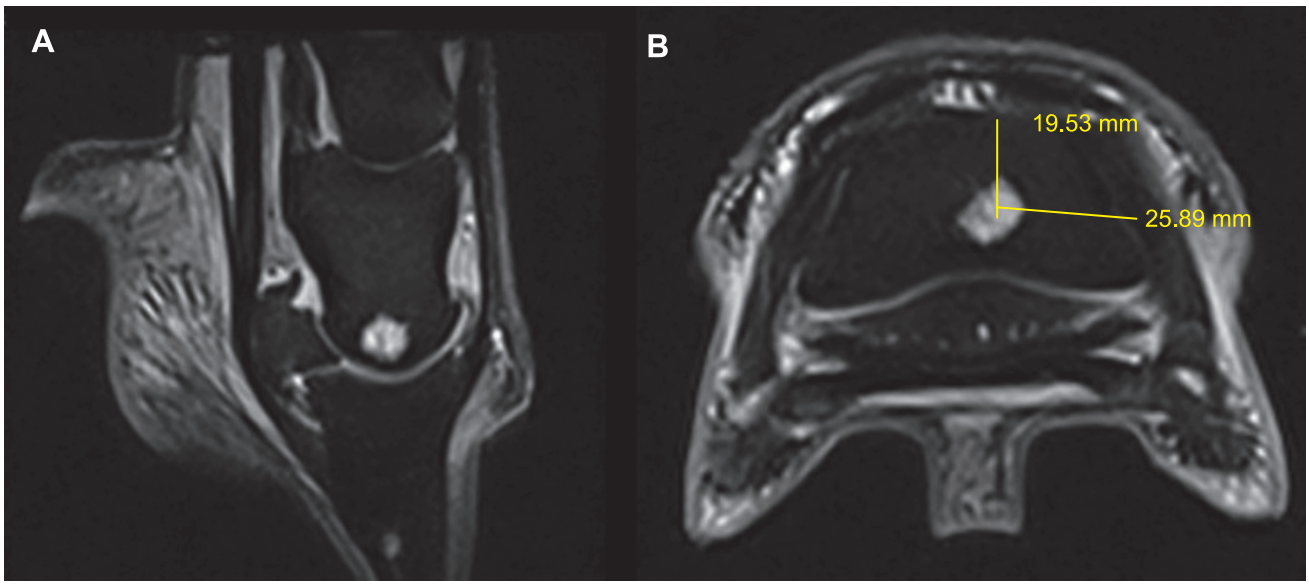

Fig. 1. MRI visualisation of the left front distal limb in STIR projecton in sagittal (A) and transverse (B) plane presenting SBC located centrally at the distal part of short pastern bone (P2). Measurements were made prior to surgery while planning surgical approach.

surgical approach but also as a valuable source of information concearning joint condition and better information for the prognosis (Figs. 1, 2). The examination was performed under general anaesthesia, with an analogous anaesthesia pattern as described in the surgical approach method. The machine used was a low-field Esaote O-Scan Equine MRI system providing a power of 0.35 Tesla.

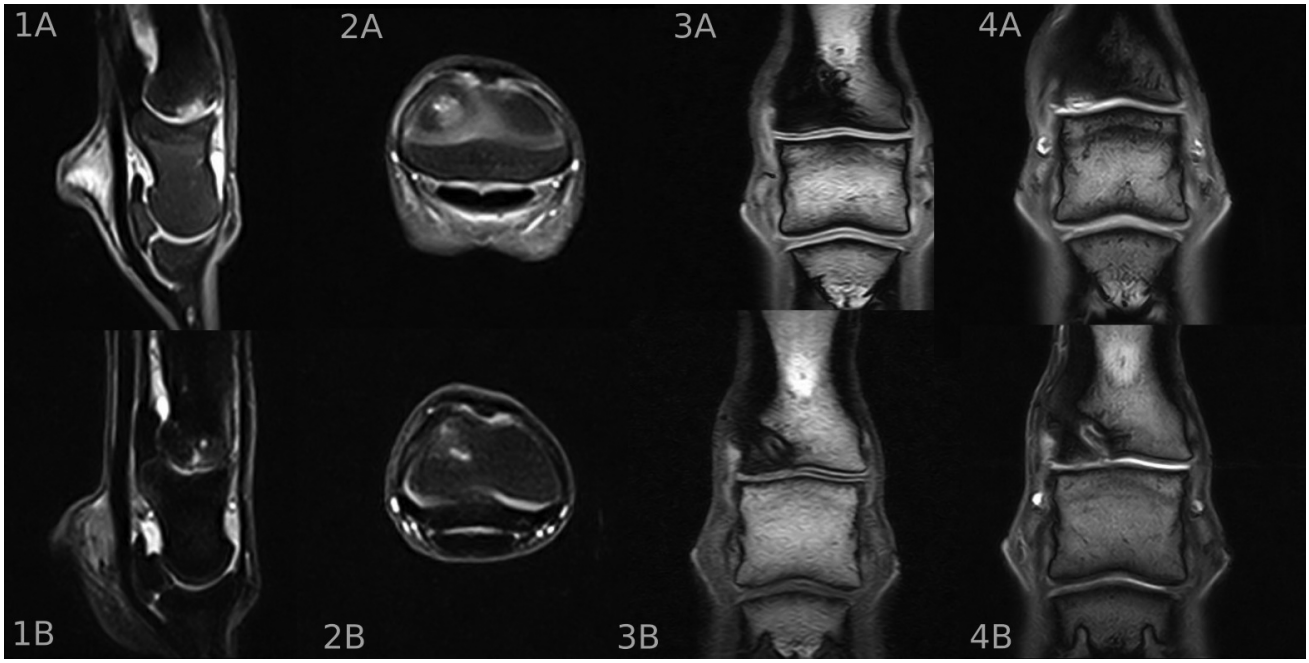

Fig. 2. A: Preoperative images presenting fluid accumulation (STIR 1,2) due to bone oedema connected with a subchondral bone cyst formation at the distal aspect of the long pastern bone (P1) in the medial condyle with accompanying bone sclerosis concerning the medial condyle of the distal aspect of P1 and proximal aspect of short pastern (P2) with a loss of trabecular bone architecture and demineralization (SD SST1, 3)followed by some minor osteoarthritic changes present in the pastern joint and severe articular cartillage damage (3D SHARC 4). Additionally remodelling of the proximal origin of the collateral medial ligament has been noted due to architectural changes of the bone together with joint instability (2, 3).
B: Images taken 3 months post-operatively, hyperechogenic pattern visible due to surgical drill usage. Scans present effective healing stage with a significant reduction of fluid accumulation in STIR images (1,2) and architectural changes in bone tissue leading to trabecular bone reconstruction together with smoothening of the periosteal bone at the origin of collateral medial ligament of the pastern joint (3) and articular cartillage reconstruction (4).
1- STIR images in sagittal plane, 2 - STIR images, transverse plane, 3 – 3D SST1 dorsal plane, 4 – 3D SHARC dorsal plane; 2,3,4 – medial is to the left.

Anesthesia and surgical preparation

Horses were prepared for surgery in a routine manner. Fasting began 12 hours prior to anesthesia. A dosage of 80-100 mg/100 kg of xylazine (Sedazin®, Biowet Pulawy, Poland) given intravenously was used as a sedative. Once the horse reached a sufficient sedation level a dosage of 2.2 mg/kg ketamin (Bioketan®, Vetoquinol Poland) and 0.02 mg/kg of diazepam(Relanium®, Polfa Warszawa, Poland) was administered, also intravenously. When lying down in the anesthesiological box the horse received a 25-100 mg/kg bolus of guaifenesine (Guajatal® 100 mg/ml, 500ml, Eurovet Animal Health, France) with 5-6 mg/kg of thiopental-natrium (Thiopental® 1g, Rotexmedica, Germany) until sufficient effect was achieved (this usually meant approximately 150-300 ml of the mentioned mix). After positioning the horse onto a surgical table, inhalation anesthesia was maintained using isoflurane and oxygen in a semi-closed inhalation system. The horses were positioned in dorsal or the lateral recumbency depending on the location of the SBC. Part of the leg was clipped, shaved and sterilized in preparation for surgery. The draping of the leg was done carefully using Kruuse Buster 120x250cm OP-cover® and self-adhesive 3M Steridrape® nr 1037 or 1040. After surgical preparation and draping, sterile needles were placed in the skin under radiographic control to determine the optimal place for skin incision with respect to the prospect-drilling axis.

Surgical technique

The stab incision in the skin was made just above the bone avoiding ligaments and the extensor tendon. To determine the appropriate angle and orientation of the bit on the drill preoperative radiographs were taken (Fig. 3A). This meant that the bit should hit the cyst without drilling into the joint space. In one of the cases three channels were created using one skin incision and drilling was done in a few slightly different directions, as the size of the cyst required this. In the case of the cyst lying in the dorso medial part of the radial proximal epiphysis, the skin incision was done medial to the border of the brachial muscle. A tunnel was formed in the space medial to the brachial muscle and lateral to the long part of the medial collateral ligament of the elbow

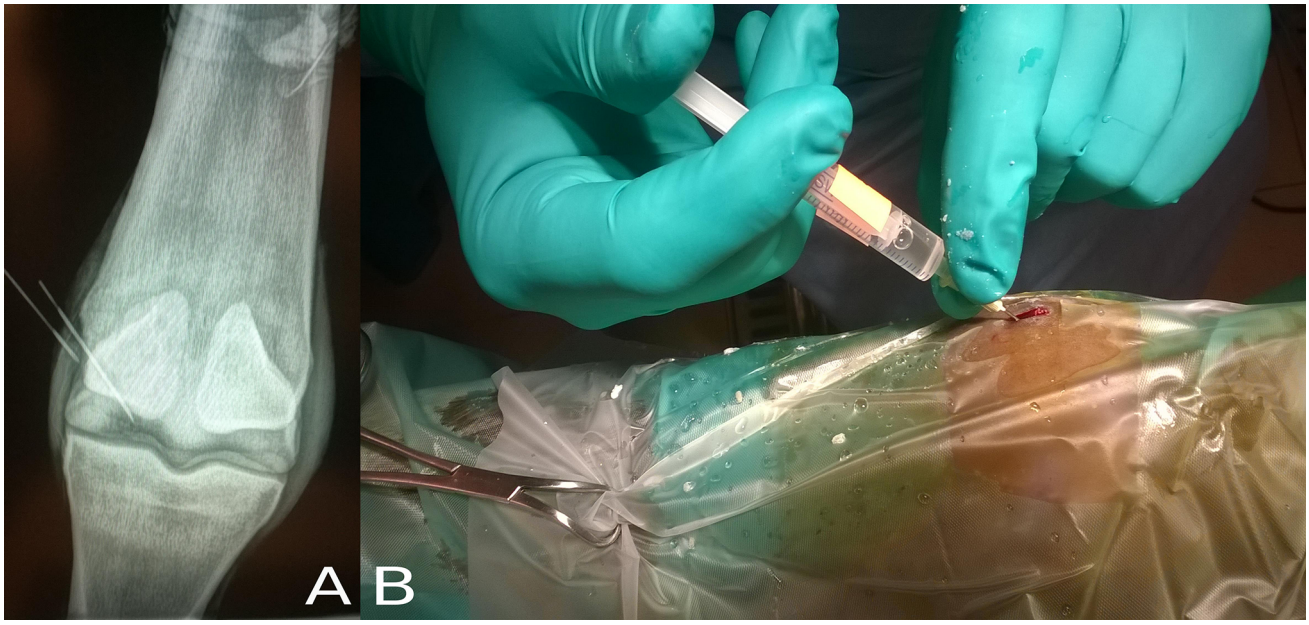

Fig. 3. A: Intraoperative control radiograph performed directly after drilling. Note: the needle was placed in the drilling hole for better evaluation. B: Administration of stem cells alginate/$CaCl_2$

joint for the drill. The horse was positioned in dorsal recumbency for this scenario. The 4.5 mm drill bit was then inserted 3 cm into the sagittal plane in the radial bone. With careful drilling, the surgeon could often feel when the drill bit had reached the empty space of the cyst. After drilling the canal and flushing of the cyst with sterile saline solution, the alginate with ASCs was injected using a syringe, with an injectable needle, through the previously drilled hole. Immediately after administration of the ASCs/alginate suspension, a $CaCl_2$ solution from a different syringe was injected to fix the stem cells in the cyst (Fig. 3B). The combination of stem cell alginate and $CaCl_2$ solutions immediately formed a soft but stable mass. This was important in order to repeat the procedure a few times, starting from the bottom of the cyst. The quantity of fluid used for filling the cyst varied between 1 to 2 milliliters. In a few of the cases, the mass of stem cells alginate and $CaCl_2$ protruded above the bone and had to be carefully cut out using a scalpel blade, as pulling it with any instrument could have removed all the contents from the cyst. Once administration of both substrates was complete, the surgery was finalized by suturing the skin incision with a single mattress suture using monofilament 3.5 or 4 metric nylon threads (Monosof®, Covidien). In the case of radial bone SBC, the subcutis layer was done using 3 metric Polyglactin 910 (Polysorb®, Covidien). A sterile bandage dressing was placed over the incision site.

Postoperative management

Postoperative antibiotics were optional but in the cases of distal P2 subchondral bone cyst, of distal Mc III epiphysis and of proximal radial SBC a dosage of 6.6 mg/kg gentamicin (Gentamycyna®, Biowet Puławy, Poland) was administered intravenously for five days. Pain was controlled by the administration of flunixin meglumine (1.1mg/kg i.v. Vetaflunix®, VetAgro, Poland) between 5-7 days postoperatively depending on the weight bearing. Sterile dressing of the leg was done for 14 days after surgery and changed every 3-4 days. All horses were stall-rested for 3 weeks after the surgery. Once stall rest was complete, all horses were walked in hand for 5 minutes, twice a day. Control radiographs were taken on day 14 and then every 30 days from thereon (on average) (Fig. 4). Lameness in trot was monitored starting from 8 weeks after surgery.

Sodium Alginate Hydrogel Preparation

Sodium alginates were dissolved in 0.9% NaCl and the solutions were filtered through 0.45 μm and 0.22 μm syringe filters. For the experiments, hydrogels were composed of 2% pure sodium alginate acid solution, which was polymerized by the addition of a sterile 10 mM $CaCl_2$ solution.

Isolation of equine mesenchymal stem cells

Mesenchymal stem cells were isolated from subcutaneous adipose tissue of the horses. Fat biopsies were collected from the peritail region approximately two weeks prior to the surgery. The tissue was washed with Phosphate Buffered Saline solution (PBS) supplemented with 1% antibiotic-antimycotic solution (penicillin/streptomycin/amphotericin B; P/S/A) and cut with sur-

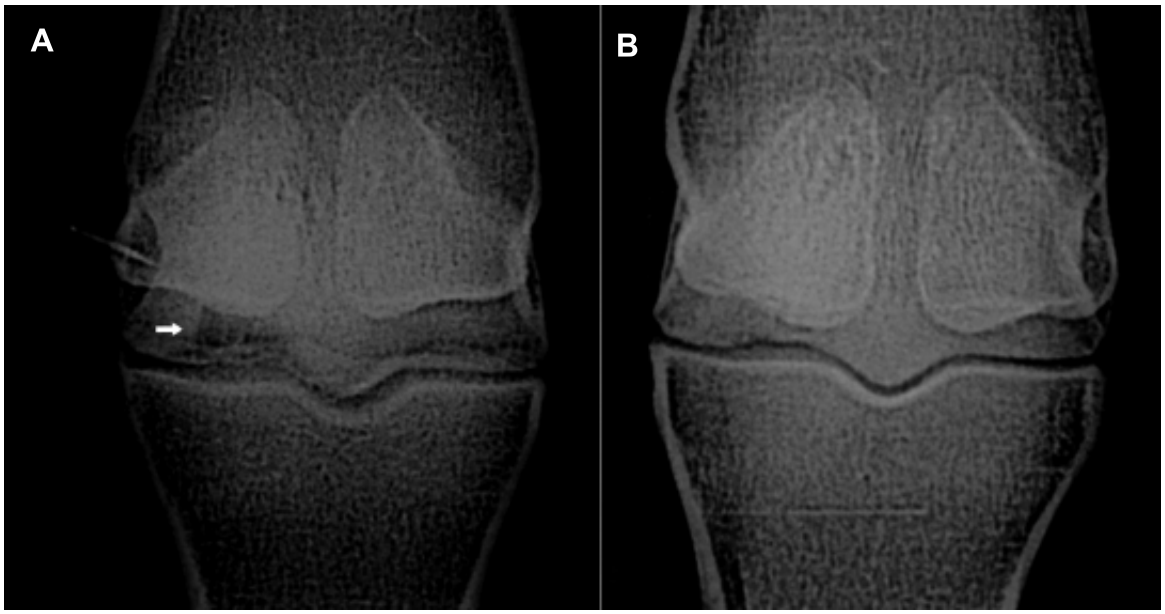

Fig. 4. Radiographic appearance of a subchondral cystic lesion in distal epiphysis of Mc III in thoroughbred colt before (A) and 5 weeks after surgical treatment (B) with the method described. Radiographs performed in a dorso-plantar projection. Lucent, flat cystic lesion marked with an arrow on intra-operative radiograph (A).

gical scissors into small pieces. The tissue was then digested in collagenase type I solution (1 mg/ml) for 40 minutes at 37°C. After centrifugation (12000 g for 10 minutes) the supernatant was discarded, the pellet containing the cells was re-suspended in culture medium, and the solution was then transferred into a cell culture flask. Adipose derived mesenchymal stem cells (ASCs) were cultured at constant conditions in an incubator (37°C, and 5% CO_2) in DMEM (Dulbecco's modified eagle's medium) containing 4500 mg/l glucose complemented with 10% FBS (Fetal Bovine Serum) and 1% of PSA was used. The media were changed every 2 days and cells that adhered to the flask were detached using TrypLE™ Express (Life Technologies, Warsaw, Poland); after reaching 80% confluence the cells were passaged three times to prepare them for the experiment.

Cell culture

Isolated cells were characterized by checking the expression of the following surface markers: CD44, CD45, CD90 and CD105. Cells were analyzed using a Becton Dickinson FACS Calibur flow cytometer. Multipotency of isolated ASCs was confirmed by osteogenic, chondrogenic and adipogenic differentiation of cells cultured in STEMPRO® Differentiation kits (Life Technologies). Cultures cultivated in standard growth medium were used as a control, in order to establish the effectiveness of differentiation. To evaluate the results of the differentiation process, cells were fixed with 4% ice-cold paraformaldehyde (PFA) and the following specific stains were performed: the extracellular mineralized matrix was visualized with Alzarin Red dye; the formation of proteoglycans was confirmed with Safranin O.; and the intracellular lipid droplets were stained red with Oil Red O.

Cell morphology was evaluated using an epi-fluorescent microscope (Axio Observer A.1, Zeiss). After fixation and permeabilization,actin filaments were stained using atto-488-labelled phalloidin at a dilution of 1:800 for 40 minutes at room temperature. Pictures were taken using a Cannon PowerShot digital camera.

Cell growth rate was evaluated using TOX-8 (Sigma Aldrich) 10% resazurin-based dye following the manufacturer's protocols. In brief, the culture media were replaced with medium containing 10% of the dye. The cells were then incubated at 37°C for 2 hours. The absorbance of the supernatants was then measured at a wavelength of 600 nm for resazurin, and at 690 nm as a reference wavelength. The number of cells was estimated on the basis of a standard curve, generated during the experiment. To prepare the curve, cells were seeded at a density of $20x10^3$, $40x10^3$ and $80x10^3$ per well and dye absorbance was measured in relation to certain cell numbers. The linear trendline equation allowed for the estimation of cell number throughout the experiment.

Analysis of gene expression: Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Cells cultured both in alginate gels and normal conditions were homogenized using 1 ml TRI Reagent.

Total RNA was isolated using the phenol-chloroform method previously described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987, Suszynska et al. 2007). Genomic DNA digestion and cDNA synthesis were performed using a PrimeScript™ RT Reagent kit with a gDNA Eraser (Takara). For each reaction, 150ng of the total RNA was used. The qRT-PCR reactions were performed using a CFX Connect™ Real-Time PCR Detection System (BioRad). Real-time PCR was also done using the SensiFast SYBR and Fluorescein Kit (Biolone) and the reaction mixture used contained 2 μl of cDNA in a total volume of 20 μl. The concentration of primers in each reaction was equal to 500nM, then the following gene expression analysis (Qn) was executed and calculated for Aggrecan, Collagen-1, bone morphogenetic protein-2 and osteopontin relative to the GAPDH housekeeping gene expression.

Statistical analysis

All experiments were performed in triplicate or more. Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, USA). Differences between groups was determined using the unpaired Student t-test. Differences with a probability of $p<0.05$ were considered significant.

Results

Initially nine horses diagnosed with subchondral bone cyst lesions, in long bone epiphysis involved in narrow joint creation were admitted to the study. One of the horses (an 11-year old warmblood gelding) with SBC located in proximal P1, had a previous 18-month history of lameness (grade 3/5 to 4/5). The cyst was not detected on radiographs taken in 2014, but radiographs taken one year later (2015) showed the presence of a large SBC in the sagittal plane of the proximal epiphysis of P1. Whilst highly recommended, the MRI examination was not performed prior to surgery, as decided by the owner, the horse was treated using the method described above in spring 2016 without success. The lameness persisted as before surgery and the cyst was still present on the radiographs. An MRI investigation done in 2017 showed a fissure line in P1 in the sagittal plane of the fetlock joint which was not visible on the radiographs. It seems that the primary reason for SBC formation was, in this case, not OC but fissure in the proximal P1 and further focal osteolysis. Lag screw fixation with administration of biphosphonates was following the MRI. For this reason the horse was excluded from the study to standardize our research group.

The horses admitted to the study showed two different grades of lameness prior to therapy (Table 1). The lameness varied from 2-3 on a 5 point scale (2-3/5) (Table 2).The horses were of varying ages, between 8 months and 10 years. Breed distribution was as follows: three horses were thoroughbred, five horses warmblood. The joints involved with the disease were as follows: one distal interphalangeal joint – SBC in distal P2, two proximal interphalangeal joints– SBC in distal P1, 3 fetlock joints – SBC in distal Mc III, one elbow joint – SBC in the proximal radius (Table 1).

All but two of the horses showed a balanced distribution of weight on all four legs just after surgery. Improper weight bearing was noticed in Mc III distal epiphysis and radial proximal epiphysis cyst patients. Weight bearing improved within 2 weeks in both cases.

The lameness disappeared in the period of 8 weeks to 4 months post treatment. One horse with significant preoperative lameness and radiographically confirmed developed OA changes including osteoohytes formation had received triamcinolone acetonide (40mg/joint; TriamHEXAL®, Hexal, Germany) intra articularly 8 weeks after surgical treatment. This horse was pasture rested for one year post surgery and is currenntly back in showjumping training with no evidence of forelimb lameness.

Discussion

In the last few decades orthopedic treatments benefited from properly addressingbiomechanics which improve fracture healing. To provide a background of some of the current therapeutic methods dedicated to SBC treatment, some literature and techniques were reviewed and after analyzing the data two general procedures were identified: conservative and surgical. Conservative therapies concentrate on limiting the amount of activity of the horse as well as any of the following: NSAiDs administration, Tiludronate, hyaluronic acid and/or local steroid applications, benzopyron was also documented in some clinical trials to be quite efficient (Jackson et al. 2008). In the use of the conservative therapies on animal patients, there was a significant reduction in lameness but the cyst remained visible in radiological images(Jackson et al. 2008). Some of the surgical techniques that have been used in the studies we reviewed are: 1) Mettenleitner used bone cement for replacement of SBCs after drilling the bones of ulnar joints (Mettenleiter 2014), 2) Santschi stabilized the bone surrounding the cyst using lag screw fixation of the femur condyle (Santschi et al. 2015), where there was a 75% success rate in horses within 120 days (complete soundness), 3) arthroscopical techniques in combination with triamcinolone or stem cell administration and/or filling the cyst with bone cement were also described (Nixon 2010), 4) administration of

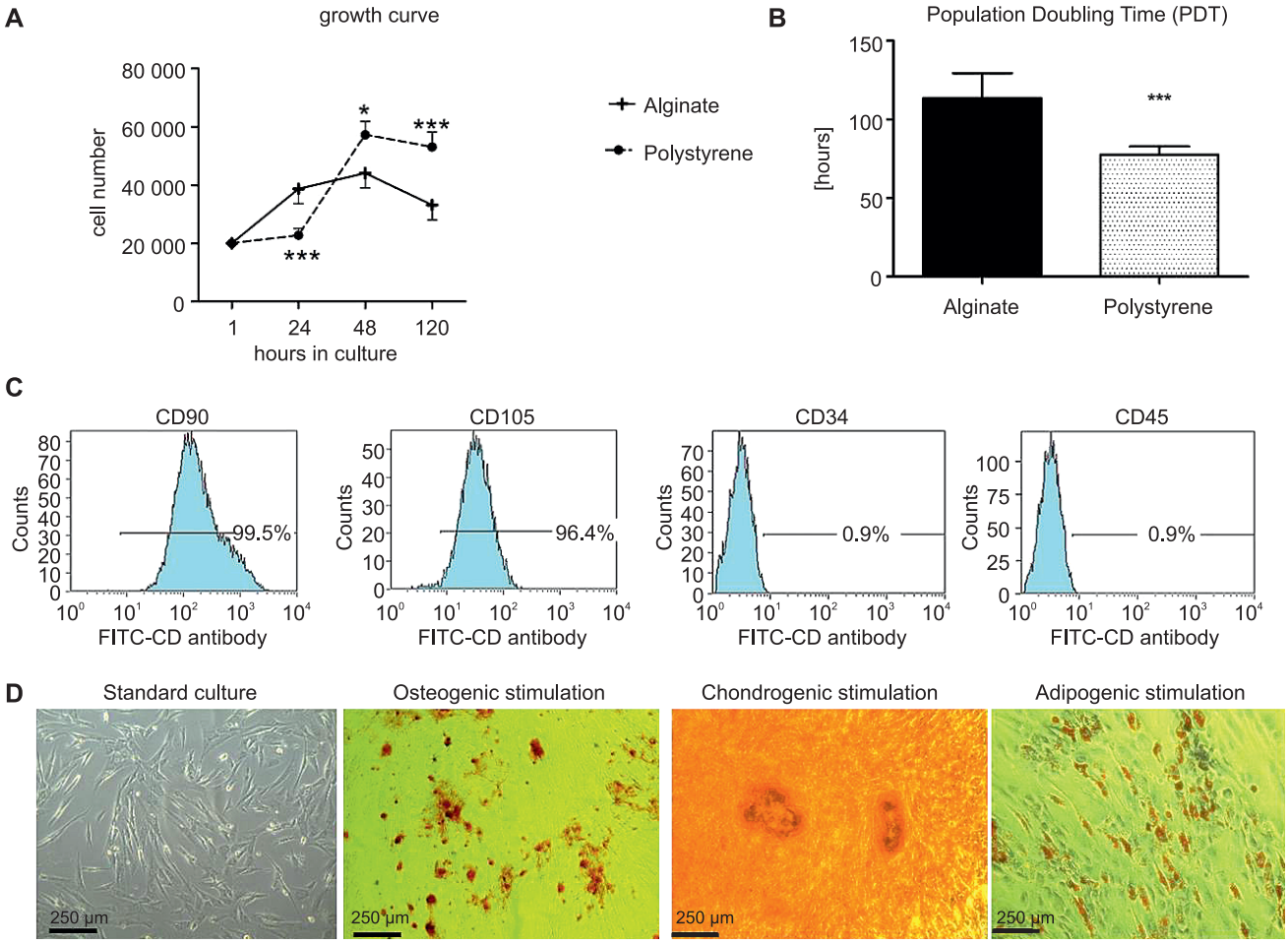

Fig. 5. Evaluation of growth kinetics (A) and population doubling time (B). Flow cytometry (C) and multipotency analysis (D) representative for equine adipose derived mesenchymal stem cells.

parathyroid hormone stabilized with fibrynous aquagel after extracapsular access, where there was an estimated 73% efficiency rate. Among the surgical methods implemented in SBC treatment, two major approaches are frequently used: arthroscopy or arthrotomy and extraarticular procedures. Originally arthrotomy incorporated with surgical debriment was a fruitful procedure, until arthroscopy came onto the market; arthroscopy less invasive, leading to minimal trauma and perfect visualization of changes, mostly in stifle joints. From there, Ortved et al. (2012) introduced an arthroscopical technique in conjunction with the administration of allogenic chondrocytes complemented with IGF – 1. Their study concluded that this technique leads to clinical improvement of the horse and therefore could be efficaciously applied in veterinary regenerative medicine.

The problem then arose as to how to deal with SBCs involving small joints, with little to no arthroscopical access. In our study all horses with SBCs in small joints showed varying grades of lameness, and older horses displayed signs of osteoarthritis (OA) in radiograph images. The inability to do effective arthroscopic surgery, or if arthroscopic surgery is performed unsuccessfully, which could lead to increases in TNF and interleukin 1 levels in the joint after surgery causing rapid development of OA and further joint cartilage damage (Fernandes et al. 2002, Kapoor et al. 2011), led us to the idea of avoiding joint irritation. Using the direct drilling technique into the cyst and filling it with the stem cell/alginate and $CaCl_2$ complex, appeared to be a logical and relatively simple surgical procedure, which presented less trauma to the horse. What is essential in our opinion is that filling the cyst should be performed without entering the joint space. SBCs are usually thought to be one of three different forms of Osteochondrosis (OC), which causes disruption in joint cartilage, surrounding bone and synovia, and contributes directly to OA. Using this method, subchondral bone cysts are replaced with normal bone tissue and thus also the changes located in the bone margin in the joint can also heal sufficiently, which induces full recovery from the disease. It seems clear that young horses with minor pathological changes in the joint cartilage and underly-

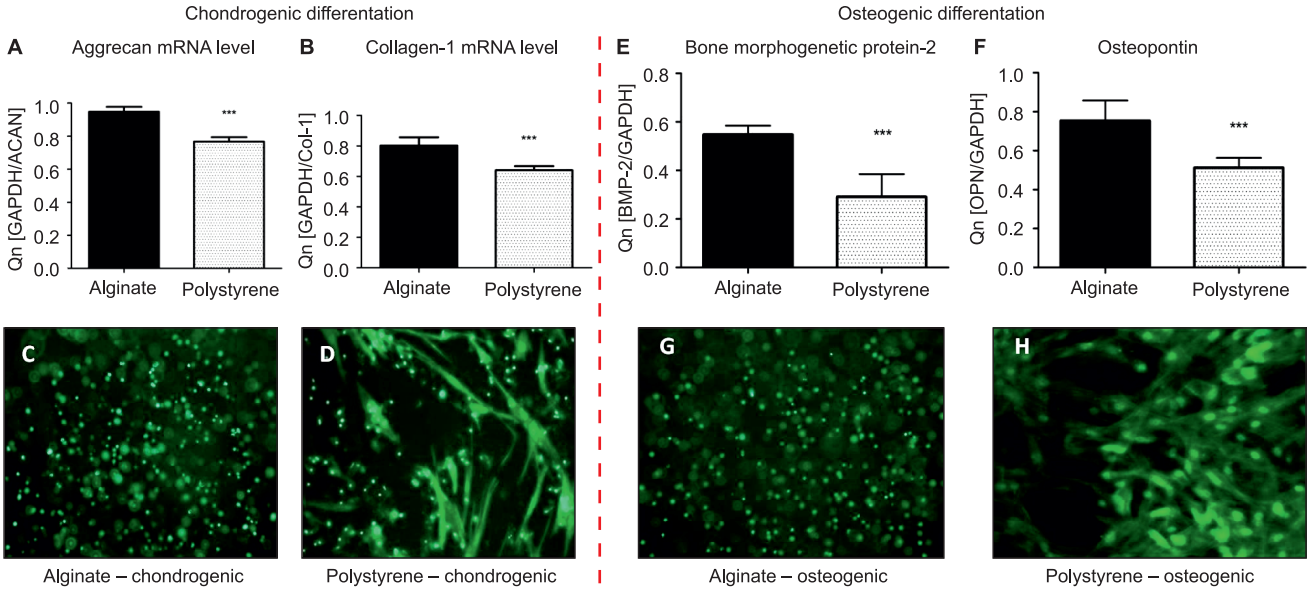

Fig. 6. Chondrogenic (A-D) and Osteogenic (E-H)differentiation of ASC after 14 days of propagation. Evaluation of protein (Agreccan-A, and Collagen-1 – B) expression during chondrogenic differentiation and BMP-2 (E) and RT-PCR results for BMP-2 (E), and Osteopontin (F). Visualization of cellular morphology Phalloidin (C, D and G, H) showed formation of cellular aggregates (x40).

ing bone surrounding an SBC are more likely to achieve soundness than older horses already suffering due to OA changes. Imbalanced levels of calcium and phosphorus as well as low levels of copper are often causes of OC development (Jeffcott and Savage 1996).

Before *in vivo* examination, we performed *in vitro* analysis of cell morphology and proliferation rate of ASCs cultured in sodium alginate hydrogels. Results of analysis indicated a lower proliferation rate and population doubling time of cells cultured in sodium alginate hydrogels over 120 h of culture, but sodium alginates allowed cells to adhere to each other and maintain normal morphology (Fig. 5A, B). The mesenchymal character of cells used in the study was also confirmed by observation of the multipotential character of cells and FACS analysis, which confirmed the presence of characteristic surface markers (Fig. 5B, C). At the same time we observed better osteogenic (BMP-2, Osteopontin) and chondrogenic (agrecann, colagaen type 1) differentiation properties of cells cultured in the investigated hydrogel in comparison to cells cultured on polystyrene wells in normal conditions. The differentiation properties of cells we confirmed via gene expression analysis and microscopic observations (Fig. 6).

During *in vivo* experiment we treated SBCs in the stifle (femur condyle) using arthroscopy, debriment, lavage and administration of triamcinolone. In two cases the arthroscopy of the stifle joint was done for the second time after 8 and 12 weeks respectively. The debris was present again in the cyst, ndicating failure of this type of therapy. Instead of this therapy we decided to experimentally fill the SBC in the femur condyle with stem cells/alginate suspension and $CaCl_2$. The gait improvement was rapid and control radiographs performed after the surgery showed progressive reduction of SBC size.

Concerning our research group three Thoroughbred yearlings (colts) did not require any further treatment and two of them treated in 2015 are currently in race training, while the third will start training in autumn 2017.One horse from the group required a longer recovery due to OA changes. In this horse (a 9-year old KWPN showjumping stallion) the grade of lameness decreased from 3/5 to minimal irregularity six months after surgery with the limited fetlock flexion test. One year after surgery the stallion started training again. The SBC healed successfully (MRI control investigation performed) but it seems that changes in joint cartilage and OA due to the long-term presence of SBC in McIII are responsible for a longer recovery period.

Conclusion

Although one horse required longer recovery due to OA changes, all patients treated with introduced method are currently sound without any sign of SBCs on radiographs. Good treatment results in the authors' belief allows for the acknowledgement of the treatment of subchondral bone cysts described above as a valuable prospective procedure for standard use.

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