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The Autologous Gelsolin Combined with Exogenous Nucleotides enhance Chondrogenic Differentiation in Equine Adipose Derived Mesenchymal Stromal Cells - An *In Vitro* Research

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Abstract

Background: Nowadays, veterinary practice, involves treatment of disorders associated with cartilage degeneration using advanced therapy, based on mesenchymal stromal cells application. Preparation of cells for the transplantation requires in vitro culture and evaluation of their chondrogenic potential.

Purpose: To determine if autologous gelsolin derived from equine serum and exogenous nucleotides may enhance the metabolic activity and chondrogenic differentiation of equine adipose derived mesenchymal stromal cells (EqASCs).

Methods: Mesenchymal stem cells were isolated from equine subcutaneous fat tissue.

Standard growth medium was supplemented with 1% of gelsolin or/and 0.1 mg/ml of nucleotides. Proliferation activity of the cells was determined basing on results obtained with cytotoxic assay and analysis of microvesicles shedding. Morphology, cytophysiological activity and chondrogenic differentiation potential were evaluated utilizing light, fluorescent and scanning electron microscopy. The effects of chondrogenic stimulation were determined via (i) analysis of gene expression for cytoskeleton and matrix proteins; (ii) proteoglycan histochemistry and (iii) analysis of culture growth pattern and chondro-nodule formation.

Results: The enhancement of proliferation activity was noticed in cultures stimulated with exogenous nucleotides. Although the addition of gelsolin did not affect EqASCs proliferation, it contributed to cytoskeleton integrity. Both investigated factors positively influenced on chondrogenic differentiation – gelsolin through stabilization of cytoskeleton proteins expression, while nucleotides by promoting nodules formation. Combination of gelsolin and nucleotides enhanced cells proliferation, what was reflected by higher cellular activity and microvesicles shedding and influenced on chondro nodules formation.

Conclusions: Autologous gelsolin and exogenous nucleotides implemented in the in vitro cultures of mesenchymal stromal stem cells isolated from adipose tissue may enhance cellular integrity, proliferation and chondrogenic differentiation of those cells, thus directly might influence on regenerative potential of adipose derived mesenchymal stromal stem cells transplants used for equine osteoarthritis treatment.

Keywords: Gelsolin; Exogenous nucleotides; Adipose-derived stromal cells; Chondrogenesis

Introduction

Adipose derived stromal cells isolated from adipose tissue (ASCs) have been used in regenerative medicine for several years, both in humans, as well as in animals. Great proliferative potential of ASCs is reflected in remarkable results of therapies based on autologous or allogeneic ASCs' transplantations. The main target area of regenerative medicine is degenerative joint diseases, especially equine osteoarthritis (OA)–the main reason of lameness development [1-5]. Osteoarthritis is associated with degradation and loss of physiological function of cartilage. The articular cartilage has limited ability for self-renewal, therefore in the context of cartilage restoring, an intense proliferating activity of ASCs is a highly desirable feature. Moreover, ASCs exhibit high cellular plasticity, and may differentiate into cells of mesenchymal lineage, namely chondrocytes [6,7]. This attribute, along with the self-renewing properties, makes ASCs a promising candidate for advanced cell-based therapy of cartilage defects.

Differentiation and regenerative potential of ASCs are features associated with autocrine and paracrine signaling, which involve microvesicles (MVs) shedding [8]. Microvesicles are small membrane bound fragments, rich in a broad spectrum of grow factors, such as VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) or transforming growth factor β (TGF-β). These highly biologically active agents affect the potential for proliferation and differentiation of mesenchymal stem cells [9] and are involved in suppression of local inflammation and immune response, as well as

inhibition of apoptosis at the site of tissue injury [10]. Additionally, MVs can be absorbed by neighboring cells, they are considered as the important mediators of cellular communication [11,12].

Further, proliferation and differentiation processes of ASCs might be affected by various endogenous mediators secreted by cells. Currently gelsolin draw attention as a protein which endogenous synthesis is important for morphogenesis and organism homeostasis as it prevents apoptosis [13].

Gelsolin belongs to a family of actin remodeling proteins and constitutes a founding member for this group of molecules. This multifunctional factor affects cell shape by regulating dynamics of actin filaments [14-16] and the rearrangement of cytoskeleton [17-21]. Moreover gelsolin acts as a chemoattractant which initiates the migration of cells for example neutrophils, important in the process of tissue regeneration and repair, [22]. What is more, GSN regulates interactions between chondrocytes and modulates the biosynthesis of extracellular matrix [17-19]. As it was shown, gelsolin, along with the vimentin and decorin is considered as a marker of chondrogenesis [23,24]. The pro-regenerative properties of gelsolin have recently been recognized as effective in equine lameness treatment [25].

Beneficial effect on cells proliferation and differentiation, and thus on regenerative potential may also have nucleotides synthesized by the cells. Every injury causes an increased activity of cells, which among others, may respond by releasing the nucleotides and their derivatives. Extracellular nucleotides bind to plasma membrane and initiate intracellular signaling cascades, which lead to the changes in cell functions [26-34]

Bearing in mind mentioned above facts, we decided to determine what influence the autologous gelsolin combined with exogenous nucleotides have on proliferation and chodrogenic differentiation of equine adipose-derived mesenchymal stem cells (EqASCs). The approach used in the research to determine metabolic activity of EqASC included analysis of their morphology, proliferation rate and microvesicles shedding. We established a complex approach, which enables obtaining information about ASCs regenerative potential in the context of cartilage disorders treatment, - not as a separate subject but as interdependent one which enables expanding knowledge of strategies available to apply during preparation of ASC for clinical application.

Materials and Methods

Subject

This research was conducted using tissues from one healthy and adult male horse. The study was approved by the II Local Ethical Committee, located at the Wroclaw University of Environmental and Life Science, Wroclaw, Poland. Basic reagents were obtained from Sigma Aldrich (Germany), unless stated otherwise.

Isolation of adipose-derived stromal cells and immunophenotyping

Subcutaneous adipose tissue was collected from the tail base area, according to standard surgical procedure, using local anesthesia induced by 2% lignocaine (Polfa S.A., Poland) [35]. The total amount of collected tissue samples was equal 3 grams. Obtained biopsies were transferred into a sterile tube containing Hanks' Balanced Salt Solution (HBSS) with the addition of 1% antibiotic-antimycotic solution (penicillin/streptomycin/amphotericinB). In order to remove red blood cells and to exclude microbial contamination, just before the isolation, the tissue samples were additionally washed in HBSS containing 1% antibiotic-antimycotic solution. After rinsing, the tissue was carefully cut with surgical scissors. Samples were transferred into conical tubes and digested with collagenase type I solution (0.1 mg/ml). The incubation with the enzyme solution was carried out for 40 minutes, and to ensure complete disintegration of tissue, samples were placed into CO2 incubator (37°, 5% CO2). After digestion, tissue homogenate was centrifuged at 1200×g for 10 minutes. The supernatant was discarded, whereas the cell pellet was re-suspended in culture medium. Prior to the experiment, ASCs cultures were passaged three times using trypsin solution (TrypLETMExpress; Life Technologies, Poland) according to the manufacturers' instruction. The presence of specific antigens for ASCs, i.e., CD29, CD44, CD73b, CD105 was analyzed using primary antibodies (all purchased in Sigma Aldrich, except for anti-CD44, derived from R&D Systems, UK). Hematopoetic origin was excluded by negative staining for CD45.

Propagation of equine adipose derived mesenchymal stromal cells

According to previous studies [35] the first 24 hr after isolation equine stem cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with Nutrient F-12, whereas DMEM containing 4500 mg/L of glucose was applied for the secondary cell culture. The media were supplemented with 10% of fetal bovine serum (FBS), and 1% of antibiotic-antimycotic solution. The culture medium was changed every two days. Cells were propagated in 25cm2 culture flask (Nunc) and maintained in CO2 incubator (temperature: 37°C, con 5% of CO2 and 95% humidity). When reaching approximately 80% of confluence, the cells were detached from the culture flask using trypsin solution.Prior to the test, the cells were passaged three times.

Biologically active factors

Gelsolin: Autologous gelsolin was obtained from the equine blood using GOLDIC kit (Arthrogen GmbH, Germany), in accordance with the method described by Schneider and Veith [25]. The procedure included: (i) collecting venous blood into three syringes containing gold nanoparticles (provided with the kit) and (ii) incubation of samples at 37°C in CO2 incubator in a horizontal position for 24 hours. After the incubation samples were centrifuged at 1500×g for 10 minutes. Serum, obtained after centrifugation was collected and mixed into one sample of volume equal ~12 ml. Serum rich in gelsolin was aliquoted and frozen for the experiment.

Determination of gelsolin serum level: The total serum concentration of gelsolin was determined with the enzyme-linked immunosorbent assay (ELISA kit; Cusabio, Wuhan, China). For the analysis three batches were selected randomly from the pooled serum. All steps of the ELISA test where performed in accordance with the manufacturer's protocol: the selected specimens were diluted 2500-times, each sample was prepared in triplicate, and spectrofotometric determination was performed using microplate reader (Spectrostar Nano, BMG Labtech) at wavelength equal to 450 nm and with the correction wavelength of 540 nm.

Nucleotides: Purified nucleotide mixture, BIORACING® derived from Greenvalley International B.V. (Netherlands). Specimens were dissolved in a sterile physiological saline solution prior to adding to the culture medium.

Analysis of equine adipose derived stromal cells' proliferation activity and morphology in the presence of biologically active factors

For the analysis cells were propagated in 24-well culture dishes, inoculated at concentration equal to 3×104 cells per well. Propagated cells were stimulated with: (i) 1% of gelsolin, (ii) 0.1 mg/ml of BIORACING® and (iii) the combination of 1% gelsolin and 0.1 mg/ml of BIORACING®. Non stimulated cells were used as a reference to the assay.

Cell proliferation assay: Proliferation activity of EqASC was determined with colorimetric cytotoxicity assay using resazurin dye. The procedure was conducted accordingly to the manufacturer' s instruction and involved: (i) removal of the culture medium and its replacement with a medium containing 10% of resazurin dye, (ii) incubation of the cells with a dye in a CO2 incubator for 2 h, (iii) collection of the supernatants and (iv) spectrophotometric measurement of the sample. Bioreduction of the dye was determined at wavelength of 600 nm and with a reference wavelength of 690 nm using a microplate reader (Spectrostar Nano, BMG Labtech). Performed analysis included a blank, i.e. a sample containing the complete medium without cells. The proliferation activity of the cells was expressed as a proliferation factor (PF), calculated accordningly to the method established previously [36,37]. Proliferation of the stimulated cells was referred to the activity of the unstimulated cells. Increase of the cells metabolic activity in tested cultures was showed as values above (PF>1), whereas its decrease as value under (PF<1) normalized unit, which reflected the proliferation activity of the control culture.

Morphology evaluation: Morphology of EqASCs was performed using fluorescent and scanning electron microscope (SEM). Evaluation of EqASCs cytology with a fluorescent microscope included analysis of mitochondria and nuclei localization, as well as determination of cytoskeleton development.

The mitochondria visualization was performed using a rhodamine-based dye, MitoRed, whereas the nuclei were stained using diamidino-2-phenylindole (DAPI) and cytoskeleton using atto-488-labeled phalloidin. The labeling of mitochondria with MitoRED was performed intravitally, while staining of nuclei and cytoskeleton was performed after cells fixation with 4% ice cold paraformaldehyde and permeabilization of cell membranes with 0.1% Triton X-100. All procedures involving fluorescence staining was performed in accordance with the manufacturer's procedure as described previously [37,38]. The cells were observed under inverted phase contrast, with an epifluorescent microscope (Zeiss, Axio Observer A.1). For SEM observations, post-fixed cells were rinsed with distilled water and dehydrated by passing through a graded series of ethanol-water solutions (from 50% to 100%, every 10%). Dried samples were sputtered with gold and put into a microscope chamber. Morphology of the cells was determined using SE1 detector, at 10kV of filament tension (SEM, Zeiss Evo LS 15).

Number of MVs evaluation: Two-dimentional images were adjusted with a color treshold plugin. Active areas were measured using a 3-D objects counter, excluding the nucleoi surface area. The total number of microvesicles on a given image was recalculated into a number of vesicles per cell.

Chondrogenesis

Chondrogenic differentiation of equine adipose-derived stem cells was performed using STEMPRO®Chondrogenesis Differentiation Kit (Life Technologies, Poland). The stimulation of EqASC was performed in accordance with the manufacturers' instruction. The culture was maintained in 24-well dishes and the cells were inoculated at concentration of 2×104 cells per well. The chondrogenic medium was changed every three days.

Evaluation of chondrogenic differentiation: The chondrogenic differentiation of equine ASCs was evaluated with Alcian blue and Safranin O staining, specific for proteoglycans. Analysis was performed after fourteen days of chondrogenic differentiation. For staining, the cells were washed with HBSS and fixed with 4% ice cold paraformaldehyde. The cells were stained with 1% Alcian blue and with 0.1% aqueous solution of Safranin O. Additionally, the cells were stained with DAPI as described above. Microscopic evaluation of the stained cultures was performed under an inverted phase contrast epifluorescence microscope (Axio Observer A.1). Imaging of chondro-nodules was conducted with SEM, and for this purpose all of the samples were prepared in the same manner as described in the paragraph above.

Gene expression analysis – RT qPCR

Cultures after chondrogenic stimulation were rinsed with HBSS and the cells were lysed directly in culture dish with 0.5 ml of TRI Reagent. The totalRNA was isolated using a phenol-chloroform method [39].

Obtained total RNA was diluted in DEPC-treated water. The concentration of RNA preparations was determined by absorbance measured at 260 nm (A260) with nanospectrophotometer (VPA biowave II). The ratio of the absorbance at 260 and 280 nm was used to assess the RNA purity. Preparation of DNA-free RNA was performed using DNase I RNase-free kit (Thermo Scientific). For each reaction 500 ng of total RNA was used. The RNA was reverse transcribed into complementary DNA (cDNA) using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) provided with VerteKitoligo(dT)15 (Novazym). All kits were used in accordance with the manufacturer's instructions.

The sequences of primers used to amplify the selected genes using RT-qPCR are presented in Table 1. The quantitative RT-PCR was carried out in a total volume of 20 µl containing 1× SensiFast SYBR master mix (A&A Biotechnology), 2 µl of cDNA and 500 nM each of gene-specific primer. Reaction was performed using CFX Connect™ Real-Time PCR Detection System (BioRad). The cycling conditions were: 95°C for 2 minutes followed by 45 cycles of 95°C for 15 s, 55°C for 60 s and 72°C for 60 s with a single fluorescence measurement. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The melting curve program was ramped from 70 to 95°C with a heating rate of 0.2°C/s and a continuous fluorescence measurement. Theoretical melting temperatures of amplicons were determined using Oligonucleotides Properties Calculator [40] and compared with experimental data in order to verify its specificity.

The threshold cycle value (Ct) and reaction efficiency were calculated basing on relative fluorescence units (RFU) and using Real-Time PCR Data Miner platform [41]. Results were expressed as relative units in reference to the housekeeping gene expression (here: GAPDH) and including efficiency of reaction.

Gene	Primer	Sequence (5-'3')	Loci	Product length	Reference
Eq-GAPDH	F:	GATGCCCCAATGTTTGTGA	428-446	250	XM_001488655.3
	R:	AAGCAGGGATGATGTTCTGG	658-677		
Eq-β-actin	F:	GATGATGATATCGCCGCGCTC	4-24	281	NM_001081838.1
	R:	CGCAGCTCGTTGTAGAAGGT	265-284		
Eq-Decorin	F:	GATGCAGCTAGCCTGAGAGG	898-917	248	NM_001081925.2
	R:	GTGTTGTATCCAGGTGGGCA	1126 -1145		
Eq-Vimentin	F:	GCAGGATTTCTCTGCCTCTT	1371-1390	205	NM_001243145.1
	R:	TATTGCTGCACCAAGTGTGT	1554-1573		

Table 1: Sequences of primers used in the experiment

Statistical analysis

Statistical analysis was performed using the Statistica 7.0 software (StatSoft, Inc., Statistica for Windows, Tulsa, OK). The normality of the variables was determined using Shapiro-Wilk test, while equality of variances was assessed by Levene's test. Differences between groups were using one-way analysis of variance (one-way ANOVA) with Dunnett post-hoc analysis.

Results

Level of gelsolin

The concentration of gelsolin in serum, obtained from equine blood using GOLDIC kit (Arthrogen®) determined with the ELISA was equal 830 ± 55 μg per ml of serum.

Immunophenotyping of EqASCs

The analysis of ASC phenotypes using specific immunofluorescence staining showed that the population obtained expressed the following markers: CD29, CD44, CD73b and CD105. Isolated EqASCs were negative for CD45, which excluded their hematopoietic origin (Figure 1).

The morphology of EqASCs

The morphology of stem cells, isolated form equine adipose tissue was determined after 72 hours of culture. Morphological differences between cells were shown in Figure 2.

The experimental conditions of cultures did not affect negatively on cells morphology nor the growth pattern. In all investigated cultures cells exhibited fibroblast-like morphology (Figure 2A-G). Due to high confluence in cultures with gelsolin and gelsolin combined with nucleotides, cells exerted bipolar shape (Figure 2A, E and G), whereas in cultures with nucleotides cells were mainly multipolar (Figure 2F). Moreover, in the cultures where gelsolin was implemented cells formed uniform monolayer (Figure 2A), whereas the addition of nucleotides initiated formation of cellular aggregates (Figure 2B and C). However, in all experimental cultures the intracellular connections were more developed than in control culture.

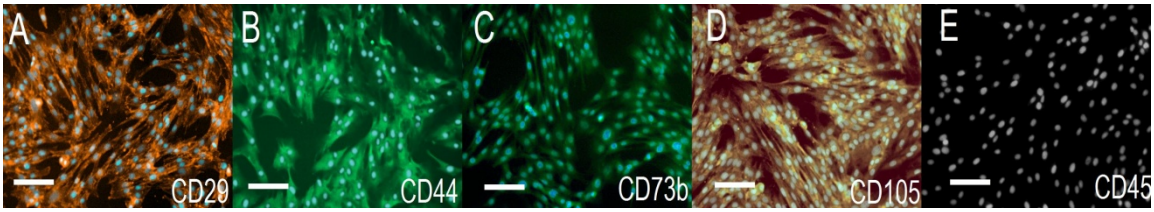

Figure 1: Analysis of ASC phenotypes using specific immunofluorescence staining showed that the population obtained expressed the following markers: CD29 (A), CD44 (B), CD73b (C), CD105 (D) and CD45 (E). Scale bars 100 μm

The nuclei of cells were located centrally. Slight signs of nuclei degeneration, expressed by forming apoptotic bodies were noted in culture with gelsolin combined with nucleotides (Figure 2C), pure nucleotides (Figure 2B), and also in non-supplemented culture (Figure 2D). The cytoskeleton was developed properly in all cultures (Figure 2E-G). The occurrence of cells with expanded body was observed in cultures after addition of gelsolin both alone, as well in combination with nucleotides. The high metabolic activity of cells was expressed by numerous mitochondria located in the perinuclear area of cell body (Figure 2I-L), as well as by the prominent release of MVs observed in all stimulated cultures (Figure 2M-U). However the highest number of secreted MVs was observed in cultures with supplementation of gelsolin and nucleotides (Figure 3).

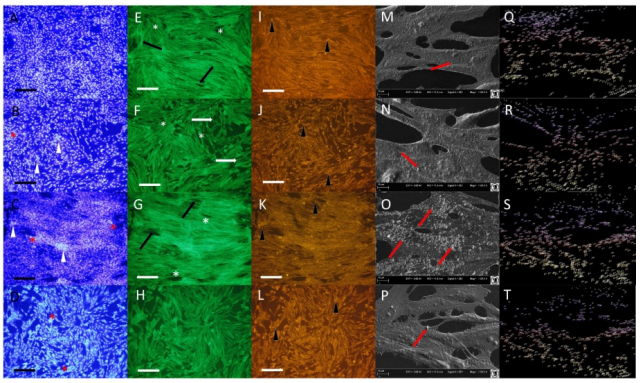

Figure 2: Cells morphology and cellular composition after treatment with 1% GNS, NT 0.1 mg/ml, GNS combined with NT and in control samples. Images taken using inverted fluorescent microscope were shown in the figures A-F. Figure A-D presents cells after DAPI staining, figures E-H after atto-488-phalloidin staining and figures I-L after MitoRed staining. Microphotographs obtained using scanning electron microscopes are presented in the figures M-P. Results of quantitative microvesicles' analysis performed with Image J are presented in the figures Q-T. For fluorescent microscope scale bars are 200 µm, for SEM scale bars are 10 µm. Multipolar cells were marked with white arrows, cells characterized by bipolar morphology were indicated with black arrows, while cells aggregates were shown using white arrowheads and proper developed cytoskeleton by white asterisks. Mitochondria were marked using black arrowheads, while apoptotic bodies were pointed out using red asterisks. Microvesicles were indicated with red arrows

Cell proliferation assay

The increased proliferation activity of EqASCs, regarding to control culture, was noted in cultures supplemented with pure nucleotides, as well as in combination with 1% of gelsolin. The proliferation of EqASCs was not affected by supplementation with pure 1% of gelsolin (Figure 4).

Evaluation of chondrogenic differentiation

The analysis of EqASCschondrogenic differentiation was performed by means of histochemicalstaining: Safranin-O and Alcian blue (Figure 5E-L). The distribution of cell nuclei and cell growth pattern was analysed using DAPI staining (Figure 5A-D).

Stimulation of cultures with investigated factors resulted in alteration of cell morphology, from fibroblast-like toward chondrocyte-like. This change was noted in all investigated cultures, despite the implemented conditions. However, microscopic evaluation showed that the process of chondrogenesis was accelerated in cultures stimulated both with pure gelsolin and gelsolin combined with 0.1 mg/ml of nucleotides. Characteristic chondro-nodules were observed in all investigated cultures. In the gelsolin combined with nucleotides conditions chondrocyte-like cells formed large aggregates, between which the fibroblast-like cells creating cellular monolayer were also present (Figure 5C, G, K and O). In the cultures supplemented with pure nucleotides cells formed smaller, but numerous nodules with no fibroblast-like cells between them (Figure 5B, F and J).

Histochemical staining revealed that cartilaginous nodules formed in cultures were rich in glycosaminoglycans (GAGs), moreover in cultures with the addition of gelsolin and gelsolin with nucleotides the glycosaminoglycans were detected in fibroblast-like cells between nodules Figure 5E and G). Moreover, the analysis of chondro-nodules in scanning electron microscope showed that in samples stimulated with gelsolin and gelsolin/nucleotides cells had rare lamelliopodia (Figure 5M and O), whereas stimulation with pure nucleotides and in control culture resulted in maintaining of cell connections with surface due to extensive cytoplasmatic projections (Figure 5N and P). What is more results of measuring the size of nodules, showed that nodules formed in culture 1%GSN+NT had the largest diameter (Figure 6).

Gene expression analysis

Product specificity was confirmed by postamplification melting curve analysis. The results of comparison of experimental and theoretical melting temperatures were presented in Table 2. Quantitative analysis of gene expression (Figure 7) showed that the addition of nucleotides to the chondrogenic cultures resulted in higher activity of actin, vimentin and decorin, however statistically insignificant. When compare to the control culture, the mRNA level of investigated transcripts was constant or slightly decreased in cultures stimulated with gelsolin and gelsolin combined with nucleotides.

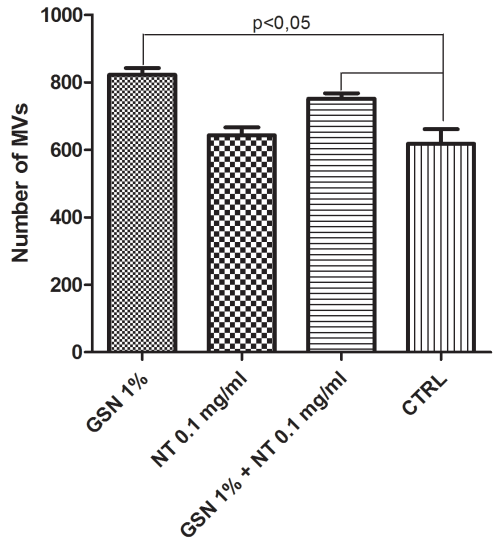

Figure 3: Number of MVs secreted from cells cultured with supplementation of gelsolin, nucleotides and both components as well as in control conditions. Marked differences were significant at $p<0.05$

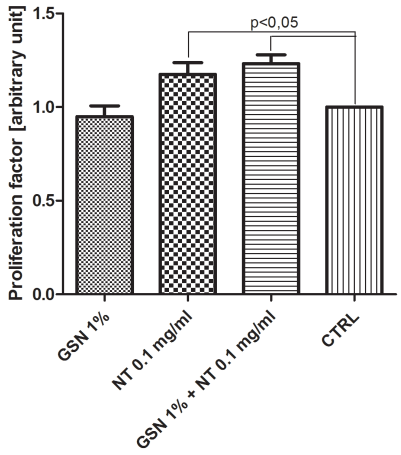

Figure 4: Distribution of proliferation ratio (mean values ± SD) evaluated for equine AdMSCs exposed to different concentrations of gelsolin and nucleotides. Proliferation 31factor (PF) was evaluated regarding to the control culture. Obtained values were shown as an arbitrary unit. Marked differences were significant at p<0.05

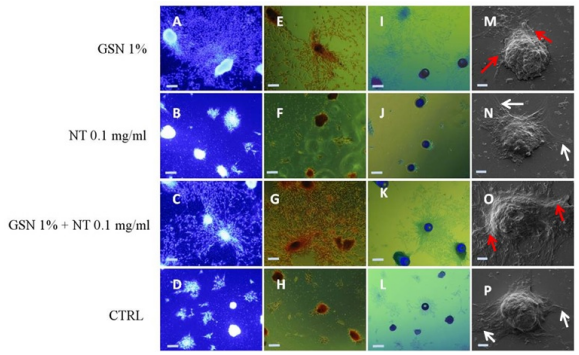

Figure 5: Chondrogenic differentiation after stimulation with 1% GNS and 0.1 mg/ml NT revealed by fluorescent and histochemical staining (respectively DAPI, Safranin O, Alcian Blue) and scanning microscope observations. Chondrogenic differentiation evidenced by the positive Safranin O and Alcian Blue staining confirming the presence of sulfated proteoglycans. SEM observation exhibited overall morphology of cartilaginous nodules. Lamelliopodia were indicated using red arrows whereas cytoplasmic projections by white arrows. For images taken by inverted microscope scale bars are 200 μm and for SEM images scale bars are 20 μm

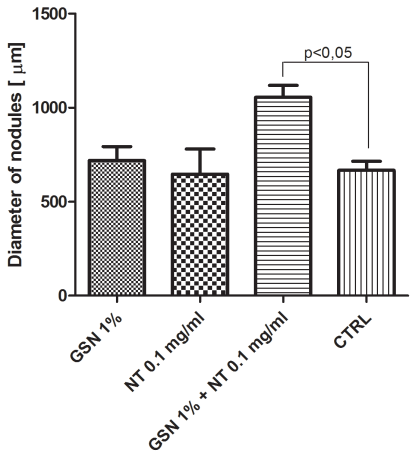

Figure 6: Comparison of nodules size in the investigated cultures. Marked differences were significant at p<0.05

Discussion

Cartilage defects and osteoarthritis are serious problems in equine veterinary medicine. The classical methods of joint degenerative diseases treatment include steroidal and nonsteroidal drugs application, however these approaches bring only temporary relief for the patients, while regenerative potential of organism is not accelerated [5,41,42]. Currently the therapies based on mesenchymal stromal cells application provide an exceptional alternative for conventional methods of OA treatment. Adipose-derived mesenchymal stem cells are increasingly used in equine veterinary medicine as a particularly promising clinical option for treating degenerative diseases and/or traumatic injuries. Additionally, features that predestinate ASCs for regenerative medicine are their proliferative potential and immunosuppressive properties. The ASCs are capable to produce wide range of cytokines and growth factors, acting both via autocrine and paracrine manner, promoting tissue restoration [42,43]. Besides self-renewal capacity, ASCs are able to differentiate into several lineages, including chondrocytes. Positive clinical effects of ASCs auto-transplantations in case of OA treatment, both in animals and humans, have been already showed [3-5]. Although therapeutic utility of ASCs has been established, the culture conditions for effective induction of chondrogenesis and for the production of a valuable cartilage are still not fully understood. The proliferation activity of ASCs in the context of transplantation seems to be crucial, as it provides the possibility for obtaining the high cell number for clinical purpose. An in vitro model applying adipose-derived mesenchymal stem cells is often used for studies regarding the chondrogenesis [44,45]. Various factors are implemented to the culture conditions in order to stimulate ASCs toward chondrogenic cells, mainly transforming growth factor-beta (TGF-β) and dexamethasone [45]. In our study the influence of autologous gelsolin and exogenous nucleotides on proliferative activity and chondrogenic differentiation of equine ASCs was investigated. Exogenous nucleotides were implemented in order to mimic the events occurring during cells degradation, associated with enhanced nucleotides releasing, whereas gelsolin was used as a factor initiating healing process. Analysis of equine ASCs proliferation showed that cells

activity was enhanced when nucleotides were implemented to the cultures. This observation correlates with analysis performed on rat fetal hepatocytes, where addition of exogenous nucleotides accelerated proliferation and maintained the quiescence of the undifferentiated cells [46].

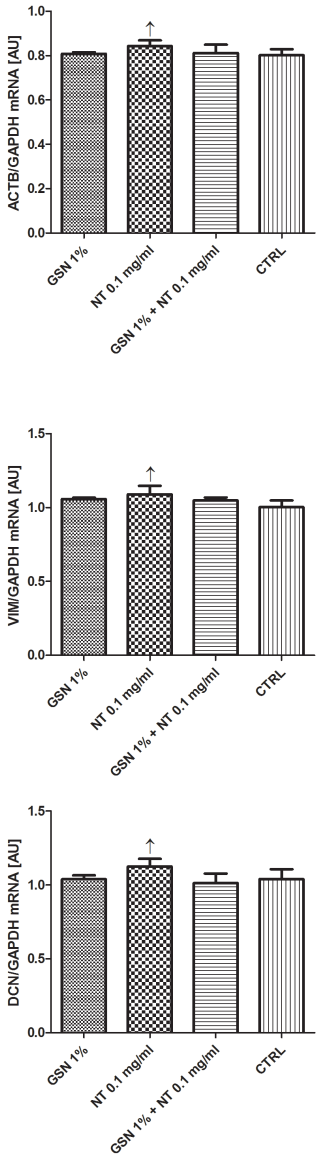

Figure 7: Expression of actin (A), vimentin (B) and decorin (C) mRNA in EqASCs in different chondrogenic culture conditions. Bars represent standard deviation of mean relative value obtained after three measurements. The arrow indicate tendency of increased expression of analyzed genes after 1%GSN+NT stimulation. The observe differences were not statistically significant.

The highest proliferation factor of equine ASCs was noticed in cultures supplemented with the combination of nucleotides and GSN. In our previous research we demonstrated the correlation between the proliferation status of EqASCs with the synthesis and release of microvesicles – which are proposed as a one of the most important factors regulating intercellular signaling [11,12]. The ASCs cultured with gelsolin and gelsolin combined with nucleotides were characterized by the highest quantity of MVs on cell surface, while in control cells and cultured with nucleotides number of MVs was lowered.

Gene	Tm [°C]	
	Theoretical	Experimental ± SD
GAPDH	88.2	88.3 ± 0.1
β-actin	95.6	94.6 ± 0.3
Decorin	83.5	83.6 ± 0.1
Vimentin	82.2	82.2 ± 0.1

Table 2: The comparison of theoretical and experimental melting temperatures of obtained amplicons

Although supplementation with pure gelsolin did not alter the proliferative activity of EqASCs, we observed its influence on cells morphology and growth pattern. As it was shown gelsolin regulates the integrity of the actin cytoskeletal structure, therefore influences on cells migration, proliferation and even ensures cells survival. Moreover gelsolin is constitutively expressed during chondrocytes differentiation; however it might be also up-regulated in hypertrophic cells [47]. The plasma concentration of GSN decreases during acute injury and inflammation, whereas application of recombinant gelsolin to animals improves recovery after sepsis or burn injuries [19,48]. Osborn et al. showed that low serum levels of GSN correlates with presence of gelsolin-actin complexes in synovial fluids, suggesting on local consumption of GSN in the inflamed joint [48]. Therefore, one may assume that injection of GSN in the site of injury may improve the healing process, due stabilization of cells proliferative activity and maintaining the structural integrity of cells.

The influence of gelsolin and exogenous nucleotides under chondrogenic conditions revealed that both investigated factors diversely impacted the differentiation of equine ASCs. While gelsolin influenced on cell morphology, causing the most evidenced alteration of EqASCs toward chondrocytes-like cells, the addition of nucleotides and combination of GSN with nucleotides resulted in enhanced formation of chondro-nodules. The effect of gelsolin shows that this protein assures appropriate cytoskeleton changes, whereas the administration of nucleotides provides aggregation of cells. Our findings are in good agreement with research performed by Ho et al. [49,50]. As it was shown the cytoskeletal proteins, actin and vimentin, influence on cellular mechanical properties, thus affecting on mechanical interactions between the chondrocytes and the surrounding tissue matrix. Analysis of gene expression, as well as specific staining confirmed stabilizing role of gelsolin both on cytoskeleton proteins (vimentin, β-actin) as well as on extracelluar-matrix proteins (proteoglycans). The lack of alteration in actin and vimentin expression after treatment with gelsolin may be very desirable feature, as these proteins play essential role in governing the solid-like viscoelastic behavior of chondrocytes, whereas instability of cytoskeleton structure could be associated with cell disintegration [51]. In all investigated cultures the histochemical staining affirmed the presence of sulfated proteoglycans, specific for the cartilage matrix. Moreover decorin mRNA was up-regulated due to stimulation with

nucleotides, thus confirming results of histochemical analysis, indicating that proteoglycans were especially abundant in nodules site.

In summary, obtained results confirmed our hypothesis that both autologous gelsolin and exogenous nucleotides positively influence on proliferation activity and chondrogenic differentiation of equine adipose-stromal cells. Due to the fact that in vitro cultures are crucial stage of preparation of clinical-grade cells, the enhancement of ASCs proliferative activity and maintenance of their proper morphology in vitro, may directly reflect on regenerative potential of implanted cells. Obtained results showed that in gelsolin and nucleotides in combination may complement each other and exert relevant effect. Compounds used in present work showed positive stimulatory effect on chondrogenic differentiation of equine ASCs in culture.

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References

1. McIlwraith CW, Frisbie DD, Kawcak CE (2012) The horse as a model of naturally occurring osteoarthritis. Bone Joint Res 1: 297-309.
2. Frisbie DD (2005) Future directions in treatment of joint disease in horses. Vet Clin North Am Equine Pract 21: 713-724, viii.
3. Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48: 3464-3474.
4. terHuurne M, Schelbergen R, Blattes R, Blom A, de Munter W, et al. (2012) Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. Arthritis Rheum 64: 3604-3613.
5. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM (2010) Mesenchymal stem cells and osteoarthritis: remedy or accomplice? Hum Gene Ther 21: 1239-1250.
6. Bruder SP, Fink DJ, Caplan AI (1994) Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem 56: 283-294.
7. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276: 71-74.
8. Marycz K, Smieszek A, Grzesiak J, NicpoÅ JE (2014) Effects of steroids on the morphology and proliferation of canine and equine mesenchymal stem cells of adipose origin - in vitro research. Acta Vet Hung 62: 317-333.
9. Marycz K, Toker NY, Smieszek A, Nicpon J (2013) The morphology and proliferation rate of canine and equine adipose derived stem cells cultured with flunixinmeglumine - in vitro. KafkasUniv Vet FakDerg 9: 1-7.
10. Orozco L, Munar A, Soler R, Alberca M, Soler F, et al. (2013) Treatment of knee osteoarthritis with autologous mesenchymal stem cells: a pilot study. Transplantation 95: 1535-1541.
11. Tetta C, Consiglio AL, Bruno S, Tetta E, Gatti E, et al. (2012) The role of microvesicles derived from mesenchymal stem cells in tissue regeneration; a dream for tendon repair? Muscles Ligaments Tendons J 2: 212-221.
12. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ (2006) Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20: 1487-1495.
13. Kusano H, Shimizu S, Koya RC, Fujita H, Kamada S, et al. (2000) Human gelsolin prevents apoptosis by inhibiting apoptotic mitochondrial changes via closing VDAC. Oncogene 19: 4807-4814.
14. Wen D, Corina K, Chow EP, Miller S, Janmey PA, et al. (1996) The plasma and cytoplasmic forms of human gelsolin differ in disulfide structure. Biochemistry 35: 9700-9709.
15. Hagiwara K, Yonedu S, Saito K, Shiraishi T, Sugo T, et al. (2005) High-performance purification of gelsolin from plasma using anion-exchange porous hollow-fiber membrane. J Chromatogr B AnalytTechnol Biomed Life Sci 821: 153-158.
16. Li GH, Arora PD, Chen Y, McCulloch CA, Liu P (2012) Multifunctional roles of gelsolin in health and diseases. Med Res Rev 32: 999-1025.
17. Ono S (2007) Mechanism of depolymerization and severing of actin filaments and its significance in cytoskeletal dynamics. Int Rev Cytol 258: 1-82.
18. Nishio R, Matsumori A (2009) Gelsolin and cardiac myocyte apoptosis: a new target in the treatment of postinfarction remodeling. Circ Res 104: 829-831.
19. Zhang QH, Chen Q, Kang JR, Liu C, Dong N, et al. (2011) Treatment with gelsolin reduces brain inflammation and apoptotic signaling in mice following thermal injury. J Neuroinflammation 8: 118.
20. Arora PD, McCulloch CA (1996) Dependence of fibroblast migration on actin severing activity of gelsolin. J BiolChem 271: 20516-20523.
21. Sun HQ, Yamamoto M, Mejillano M, Yin HL (1999) Gelsolin, a multifunctional actin regulatory protein. J BiolChem 274: 33179-33182.
22. Spinardi L, Witke W (2007) Gelsolin and diseases. SubcellBiochem 45: 55-69.
23. Djouad F, Delorme B, Maurice M, Bony C, Apparailly F, et al. (2007) Microenvironmental changes during differentiation of mesenchymal stem cells towards chondrocytes. Arthritis Res Ther 9: R33.
24. Rocha B, Calamia V, Mateos J, Fernández-Puente P, Blanco FJ, et al. (2012) Metabolic labeling of human bone marrow mesenchymal stem cells for the quantitative analysis of their chondrogenic differentiation. J Proteome Res 11: 5350-5361.
25. Schneider U, Veith G (2013) First Results on the Outcome of Gold-induced, Autologous-conditioned Serum (GOLDIC) in the Treatment of Different Lameness-associated Equine Diseases. J Cell SciTher 5: 151.
26. Bodin P, Burnstock G (2001) Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. J CardiovascPharmacol 38: 900-908.
27. Rudolph FB (1994) The biochemistry and physiology of nucleotides. J Nutr 124: 124S-127S.
28. Baldwin SA, Mackey JR, Cass CE, Young JD (1999) Nucleoside transporters: molecular biology and implications for therapeutic development. Mol Med Today 5: 216-224.
29. Holen E, Jonsson R (2004) Dietary nucleotides and intestinal cell lines: I. Modulation of growth. Nutrition Research 24: 197–207.
30. Rodríguez-Serrano F, Marchal JA, Ríos A, Martínez-Amat A, Boulaiz H, et al. (2007) Exogenous nucleosides modulate proliferation of rat intestinal epithelial IEC-6 cells. J Nutr 137: 879-884.
31. Sukumar P, Loo A, Magur E, Nandi J, Oler A, et al. (1997) Dietary supplementation of nucleotides and arginine promotes healing of small bowel ulcers in experimental ulcerative ileitis. Dig Dis Sci 42: 1530-1536.
32. Cosgrove M (1998) Perinatal and infant nutrition. Nucleotides. Nutrition 14: 748-751.
33. Gil A (2002) Modulation of the immune response mediated by dietary nucleotides. Eur J ClinNutr 56 Suppl 3: S1-4.
34. Riera J, Pons V, Martinez-Puig D, Chetrit C, Tur JA, et al. (2013) Dietary nucleotide improves markers of immune response to strenuous exercise under a cold environment. J IntSoc Sports Nutr 10: 20.
35. MarÄ™dziak M, Marycz K, Smieszek A, Lewandowski D, Toker NY (2014) The influence of static magnetic fields on canine and equine mesenchymal stem cells derived from adipose tissue. In Vitro Cell DevBiolAnim 50: 562-571.

36. Grzesiak J, Marycz K, Czogala J, Wrzeszcz K, Nicpon J (2011) Comparison of behavior, morphology and morphometry of equine and canine adipose derived mesenchymal stem cells in culture. Int.J. Morp 29: 1012–1017.
37. Marycz K, Åšmieszek A, Grzesiak J, Donesz-Sikorska A, Krzak-RoÅ› J (2013) Application of bone marrow and adipose-derived mesenchymal stem cells for testing the biocompatibility of metal-based biomaterials functionalized with ascorbic acid. Biomed Mater 8: 065004.
38. Grzesiak J, Marycz K, Szarek D, Jarmundowicz W (2013) Morphological Characterization of Gecko's (Eublepharismacularius) Glial Cells in Culture. Int. J. Morphol 31: 826-831.
39. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.
40. Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res 35: W43-46.
41. Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J ComputBiol 12: 1047-1064.
42. Goodrich LR, Nixon AJ (2006) Medical treatment of osteoarthritis in the horse - a review. Vet J 171: 51-69.
43. Fernandes JC, Martel-Pelletier J, Pelletier JP (2002) The role of cytokines in osteoarthritis pathophysiology. Biorheology 39: 237-246.
44. Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100: 1249-1260.
45. Ronzière MC, Perrier E, Mallein-Gerin F, Freyria AM (2010) Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. Biomed Mater Eng 20: 145-158.
46. Boeuf S, Richter W (2010) Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. Stem Cell Res Ther 1: 31.
47. Sáez-Lara MJ, Manzano M, Angulo AJ, Suárez A, Torres MI, et al. (2004) Exogenous nucleosides stimulate proliferation of fetal rat hepatocytes. J Nutr 134: 1309-1313.
48. Nurminsky D, Magee C, Faverman L, Nurminskaya M (2007) Regulation of chondrocyte differentiation by actin-severing protein adseverin. DevBiol 302: 427-437.
49. Osborn TM, Verdrengh M, Stossel TP, Tarkowski A, Bokarewa M (2008) Decreased levels of the gelsolin plasma isoform in patients with rheumatoid arthritis. Arthritis Res Ther 10: R117.
50. Ho WC, Greene RM, Shanfeld J, Davidovitch Z (1982) Cyclic nucleotides during chondrogenesis: concentration and distribution in vivo and in vitro. J ExpZool 224: 321-330.
51. Trickey WR, Vail TP, Guilak F (2004) The role of the cytoskeleton in the viscoelastic properties of human articular chondrocytes. J Orthop Res 22: 131-139.