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RESEARCH ARTICLE

The Morphology and Proliferation Rate of Canine and Equine Adipose Derived Mesenchymal Stem Cells Cultured with Flunixin Meglumine-*in vitro*

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Summary

The regenerative medicine in animals is a rapidly growing field, especially when therapies with stem cells are applied. Currently, stem cells are used for treatment of orthopedic diseases occurring both in small and large animals. Non-steroidal anti-inflammatory drugs (NSAIDs) are routinely used and are often accompanied with adipose derived mesenchymal stem cells (ADMSCs) therapy. Therefore, it is reasonable to monitor morphology of cells and the proliferation status of canine and equine ADMSCs cultured with NSAIDs. We focused on the analysis of the above mentioned parameters. Morphology of investigated cells was monitored using an epifluorescence microscope and scanning electron microscope (SEM). Moreover, SEM analysis was carried out for determination of microvesicles secretion. Proliferation activity of AdMSCs was evaluated with a resazurin-based test. Our research showed that the lowest concentration of Flunixin meglumine (0.01 mg/ml) had a stimulating effect on canine AdMSCs proliferation, while the same concentration significantly slowed down equine stem cell growth. Interestingly, the 0.01 mg/ml concentration of Flunixin meglumine did not effect morphology of the investigated stem cells population. Thus, results obtained from multilevel research allow us to conclude that the lowest concentration of Flunixin meglumine may be accompanied with canine adipose derived mesenchymal stem cells in orthopedic treatment.

Keywords: *Adipose-derived mesenchymal stem cells, Non-steroidal anti-inflammatory drugs, Flunixin meglumine, Proliferation rate, Morphology, Canine, Equine*

Flunixin Meglumine İle *in vitro* Kültüre Edilen Köpek ve At Yağ Kökenli Mezenkimal Kök Hücrelerinin Morfolojisi ve Çoğalma Hızı

Özet

Hayvanlarda özellikle kök hücrelerinin kullanıldığı rejeneratif (yenilenebilir) tıp hızla gelişim gösteren bir alandır. Günümüzde hem büyük hem de küçük hayvanların ortopedik hastalıklarında kök hücre tedavisinden faydalanılmaktadır. Klinik pratikte rutin olarak kullanılan non-steroidal anti-inflamatuar ilaçlar, adipoz kökenli mezenşimal kök hücresi tedavisine de dahil edilmektedir. Bu çalışmada köpek ve atlardan elde edilmiş adipoz kökenli mezenşimal kök hücrelerinin, yaygın kullanılan bir non-steroidal anti-inflamatuar ilaç (flunixin meglumine) ile kültüre edilmesinin, hücre morfolojisi ve proliferasyonu üzerine etkileri araştırıldı. Hücrelerin morfolojik takibinde epifuloresans mikroskop ve taramalı elektron mikroskobu (SEM) kullanıldı. Hücrelerin mikrovezikül sekresyonları da SEM aracılığıyla izlendi. Adipoz kökenli mezenşimal kök hücrelerinin proliferasyon aktiviteleri resazurin-temelli bir test yardımıyla değerlendirildi. Araştırma sonuçlarına göre fluniksin meglumin'in en düşük konsantrasyonlarının (0.01 mg/ml) köpeklerden elde edilen adipoz kökenli mezenşimal kök hücrelerinde proliferasyonu uyardığı; ancak aynı konsantrasyonun at kökenli hücrelerde proliferasyonu belirgin düzeyde yavaşlattığı gözlendi. Enteresan bir sonuç olarak 0.01 mg/ml konsantrasyondaki flunixin meglumine'in, incelenen kök hücre populasyonunun morfolojilerine etki etmediği belirlendi. Çok aşamalı bu araştırmadan elde edilen bulgular ışığında, köpeklerden elde edilen adipoz kökenli mezenşimal kök hücrelerinin ortopedik tedavilerde kullanımı sırasında, flunixin meglumine'in en düşük konsantrasyonlarda kullanılmasının yararlı olacağı sonucuna ulaşıldı.

Anahtar sözcükler: *Adipoz-kökenli mezenşimal kök hücreleri, Non-steroidal anti-inflamatuar ilaçlar, Flunixin meglumine, Proliferasyon oranı, Morfoloji, Köpek, At*

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for orthopedic patients that suffer from pain and inflammation. The group of drugs is officially classified by the World Health Organization (WHO) as a therapy of choice for mild pain treatment [1]. Its usage is particularly appropriate and has great importance in the postoperative period. The NSAIDs are also applied as an accompanying therapy in infectious diseases and musculoskeletal disorders, and are often indicated for potential oedema [1].

The role of NSAIDs involves limitation of prostaglandins (PGs) formation, which is responsible for inflammation, swelling, pain and fever [2]. The NSAIDs inhibit the activity of cyclooxygenase (COX) and therefore the production of prostaglandins (PG) and tromboxanes. Consequently, NSAIDs application leads to reduction of releasing inflammation mediators such as histamine and/or bradykinin [3].

The undisputed advantage of NSAIDs is non-addictive action; therefore prolonged application of non-steroidal anti-inflammatory drugs is preferable by many veterinarians. Moreover, NSAIDs do not cause sedation or respiratory depression. Numerous studies support this thesis that non-steroidal, anti-inflammatory drugs have satisfactory analgesic effect on small and large animals. However, all that glitters is not gold - some *in vivo* research and clinical observations have reported the side effects of NSAIDs application, *e.g.* gastric ulcers, nephrotoxycity and coagulation disturbance [1,3].

Musculoskeletal and locomotor system disorders in dogs and horses are the most commonly occurring therapeutic problems, which leads to limitations of physiological activities, and in extreme cases may cause serious clinical complications [3,4]. These disorders require immediate veterinary intervention and foremost, the application of all NSAIDs drugs. Nevertheless, this medical strategy may improve the patient's condition, but only for a short time. Therefore, modern veterinary medicine is looking for new treatment methods that would work efficiently and improve permanently the patient's clinical outcome. Regenerative medicine is a fast developing branch of veterinary care. Its main goal is to replace degenerative tissue and as a consequence, the whole organ, with a new forming structure that has complete functionality [5-8]. Regenerative medicine provides new solutions in the field of joint and ligament disorders treatment and gives hope as an alternative or complementation to other drug therapies. One of the most promising approaches is stem cell therapy [4-6,8-11]. Many research groups reported positive effects in cases of osteoarthritis (OA) and degenerative joint disorders (DJD) when adult stem cells were applied [8-12]. Adipose-derived mesenchymal stem cells (AdMSCs) together with those isolated from bone marrow (BMMSCs), seems to be populations that are the most often used in clinical practice. At today's level of scientific knowledge, we are still unable to answer the question of, which among those populations are more efficient in the treatment of particular disorders. However, clinical studies performed with the use of AdMSCs bring many positive effects and confirms the thesis that adipose stem cells are a sterling approach in veterinary regenerative medicine [5,8-10]. It was proven that these populations possess two essential features of stem cells i.e. multi-potential character and the ability of self-renewal of the population. Moreover, when introduced into an inflammatory environment, they significantly reduce inflammation process [6]. This mechanism was also clearly described by Gonzalez et al.[12], which showed AdMSCs acting as a natural blocker of pro-inflammatory cytokines.

Therapeutic effects of stem cells are probably related to their pararcine action. That is why evaluation of localization, size and density of mesenchymal stem-cells microvesicles (mMVs) seems to be crucial in the assessment of their AdMSCs' potential in terms of the regeneration process. As it was reported by Lai et al.[14], mMVs shed by adult stem cells are rich in a broad spectrum of growth factors and cytokines e.g. VEGF (Vascular Endothelial Growth Factor), HGF (Hepatocyte Growth Factor), FGF (Fibroblast Growth Factor), IGF-I (Insulin-like Growth Factor 1), MCP-1 (Monocyte Chemoattractant Protein-1) and BMPs (Bone Morphogenetic Proteins), which exert effects on cells in their vicinity. Additionally, evaluation of cytonemes and the presence of tunneling nanotubules are very important because it may directly correlate with particular culture physiological activity. The applying of stem cells in clinical treatment requires their detailed morphological and ultrastructural evaluation at every stage of culture [7,8].

The goal of this research was to evaluate the *in vitro* influence of commonly used NSAID - flunixin meglumine on canine and equine adipose derived mesenchymal stem cells. In this research we investigated morphology, viability and cytophisiological activity of canine and equine AdMSCs cultured with flunixin meglumine.

MATERIAL and METHODS

Ethical Approval

The experiment was conducted with the approval of Bioethical Commission, as stated by the Second Local Bioethical Commission at the Department of Biology and Animal Breeding, at University of Environmental and Life Sciences in Wroclaw, Chełmonskiego 38C, Poland (dec. number 177/2010 from 11.15.2010).

Isolation of AdMSCs

Mesenchymal stem cells were isolated from sub-

cutaneous fat tissue under local anesthesia, from dogs and horses. Owners of the animals provided proper agreement for the procedure. All stages of the procedure were performed as previously described [16,17]. Fat tissue biopsies (2 g) were washed in Hank's Balanced Salt Solution (HBSS) from tissue contaminations and digested in type I collagenase (5 mg/ml). Next, samples were centrifuged at 1200×g for 10 min. After centrifugation, stromal vascular fraction (SVF) was suspended in culture media and placed into culture dishes.

After 24 h, non-adherent cells were removed. For the primary culture, Ham's F12 medium was applied, and for the secondary culture, Dulbecco's Modified Eagle's medium (DMEM) ith 4500 mg/L concentration of glucose was used. Media were supplemented with 10% of fetal bovine serum (FBS). Additionally, 1% of antibiotic/antimycotic solution was used in the culture as a prophylaxis against potential infections. A constant condition of the culture was maintain during the experiment (5% CO_2, 95% humidity, at 37°C). The culture media was changed every two days.

Non-steroidal anti-inflammatory drug (NSAIDs)

Flunixin meglumine solution (Intervet®, 50 mg/mL, Istanbul, Turkey) was tested *in vitro* as an additive to culture medium at the following concentrations: 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL. The *in vitro* evaluation of funixin meglumine effect on canine and equine AdMSCs was performed in 24-well plates. The initial inoculum concentration was $2x10^4$ cells in 0.5 ml of medium per well. First dosages of the investigated drug were added after 6 h, when adhesion of the cells was observed. Untreated stem cells were used as a control for comparison with the investigated culture. All samples were prepared in duplicate.

The medium was changed 48 h after the cells' propagation. Morphology and viability of the cells were evaluated after 24, 72, and 120 h, on the basis of studies made by Nuzzi et al.[19].

Cytotoxicity Test

Metabolic activity of living cells was determined using resazurin - resorufin system (*in vitro* toxicology assay, AlamrBlue® assay, Invitrogen, USA). In order to perform the assay, supernatants were removed and replaced with a medium containing 10% of dye. Cultures were incubated for 2 h in CO_2 incubator. After the defined time, supernatants were collected and transferred into a microplate. Absorbance of supernatants was measured spectrophotomertically at a wavelength of 600 nm and with a reference wavelength of 690 nm. For population doubling time (PDT) estimation, a standard curve from different a range of cell concentrations ($2×10^4$, $4×10^4$, $8×10^4$ and $16×10^4$) was performed.

Morphology and Cell Activity Evaluation

Morphology of cells was evaluated under an inverted phase contrast microscope (Zeiss®, Axio Observer A.1). Additionally, scanning electron microscopy (SEM, Zeiss Evo LS 15) was applied in order to observe mMVs. For analysis of cellular composition, diamidino-2-phenylindole (DAPI) was used for nuclei staining, whereas phalloidin was used for actin filaments visualization. Prior to staining, cells were fixed with 4% ice cold paraformaldehyde for 15 min at room temperature. After fixation, cells were washed and permeabilized with 0.1% triton X-100 for 15 min at room temperature. Cells were washed again and stained with atto-488-labeled phalloidin for 30 min and then counterstained with DAPI for 5 min. Next, samples were washed three times and observed with an inverted, epi-fluorescence microscope. For scanning electron microscopy, cells were fixed in 2.5% glutaraldehyde (1:1 in DMEM) for 30 min at room temperature, then triple washed with phosphate buffered saline (PBS), and dehydrated in alcohol series (from 50% to 100% every 10%). Cells were observed with a SE1 detector, at 10 kV of filament tension.

RESULTS

Effects of Flunixin meglumine on Proliferation of Canine and Equine AdMSCs

The proliferation rate of canine and equine AdMSCs, depended on the concentration of flunixin meglumine used in the experiment. At 0.01 mg/ml and 0.1 mg/ml concentration the drug exerted a slight or non-toxic effect, whereas application of the drug at 1 mg/ml concentration had a strong toxic effect. An increase in proliferation activity was observed only in the canine culture after 48 h of propagation in the presence of flunixin meglumine at 0.01 mg/ml concentration *(Fig. 1)*. The same concentration of the drug did not cause enhancement of equine proliferation *(Fig. 2)*. The analysis of population doubling time revealed that the application of flunixin meglumine at concentration 0.01 and 0.1 mg/ml elongated the PDT value of CaAdMSC, in respect to the control culture. In the case of EqAdMSCs culture, the PDT value after the addition of 0.01 mg/ml flunixin meglumine was approximate to the PDT of the control culture. However, expansion rate of the culture affected with 0.1 mg/ml was higher compared to the control *(Table 1)*.

Morphology and Cell Activity of Canine and Equine AdMSCs

The lowest concentration of flunixin meglumine (0.01 mg/ml) did not affect the canine AdMSCs morphology during the entire experimental period *(Fig. 3)*. Investigated cells were characterized by a spindle shape and

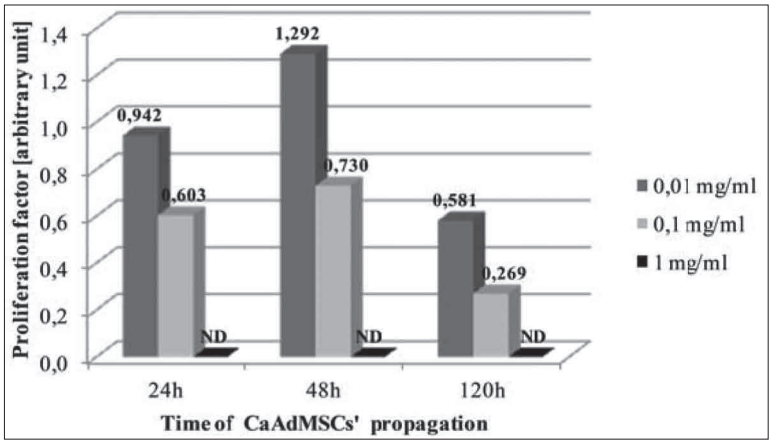
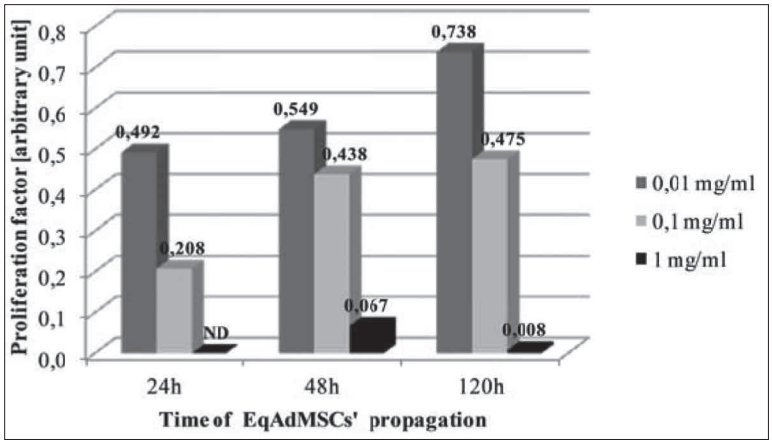


Fig 1. Proliferation ratio of canine AdMSCs treated with defined concentration of flunixin meglumine. The factor was expressed as a arbitrary unit. Abbreviation ND refers to not detectable proliferation activity of cells

Şekil 1. Değişik flunixin meglumine konsantrasyonları ile muamele edilen köpek AdMSC'lerinin proliferasyon oranı. Faktör birimi keyfi olarak belirlendi. ND hücrelerin tespit edilemeyen proliferasyon aktivitesinin kısaltmasıdır

Fig 2. Proliferation ratio of equine AdMSCs treated with defined concentration of flunixin meglumine. The factor was expressed as a arbitrary unit. Abbreviation ND refers to not detectable proliferation activity of cells

Şekil 2. Değişik flunixin meglumine konsantrasyonları ile muamele edilen at AdMSC'lerinin proliferasyon oranı. Faktör birimi keyfi olarak belirlendi. ND hücrelerin tespit edilemeyen proliferasyon aktivitesinin kısaltmasıdır



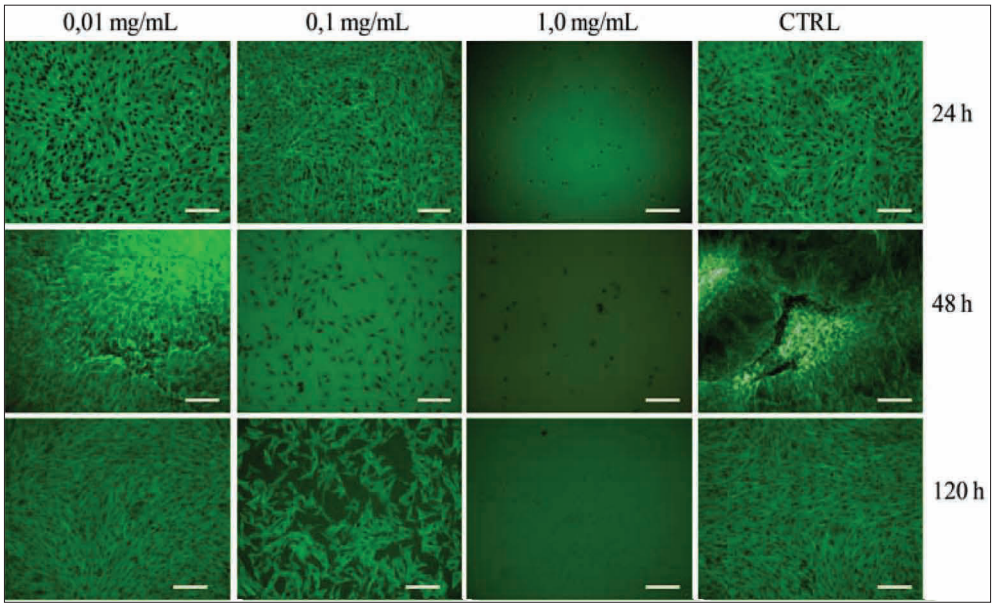


Fig 3. Morphological changes of canine AdMSCs' treated with different concentration of flunixin meglumine; Scale bar = 200 mm

Şekil 3. Farklı flunixin meglumine konsantrasyonları ile muamele edilen köpek AdMSC'lerindeki morfolojik değişimler; Ölçek birimi: 200 µm

uniform growth pattern, typical for stem cells. Higher incorporation of flunixin meglumine (0.1 mg/ml) caused equal distribution of canine cells in the culture however, single apoptotic bodies were noticed. While analyzing the highest concentration of flunixin meglumine (1 mg/ml), the first day of research revealed that apoptotic cells were predominated.

In cultures of equine AdMSCs, only the lowest concentration of the investigated drug did not affect the cells morphology. Higher incorporation of flunixin meglumine to the equine cultures induced morphological changes and promoted occurrences of apoptotic bodies on the second day of research. Untypical and incorrect morphology was observed after application of flunixin

Table 1. *The results of PDT evaluation*
Tablo 1. *PDT değerlendirilmesinin sonuçları*

Cell Culture Type	Flunixin meglumine Dosage (mg/mL)	PTD
CaAdMSCs	0.01	118.69
	0.1	347.4
	1	NA
	0	58.37
EqAdMSCs	0.01	65.38
	0.1	96.71
	1	NA
	0	70.86

meglumine at 1 mg/ml concentration. In this particular case, the presence of numerous death cells in the culture was noticeable *(Fig. 4)*.

Cell Activity Expressed by the Synthesis of Mesenchymal Microvesicles

Concentration of flunixin meglumine 0.01 and 0.1 mg/ml, had a stimulating effect on mMVs synthesis in canine and equine mesenchymal stem cells (*Fig. 5* and *6*). It was maintained until the 120th h of the experiment. The higher concentration of flunixin meglumine 1 mg/ml caused abnormal cell morphology in both species, thus scarce mMVs was observed. Also, when distribution of mMVs is considered, especially in regard to the equine culture,

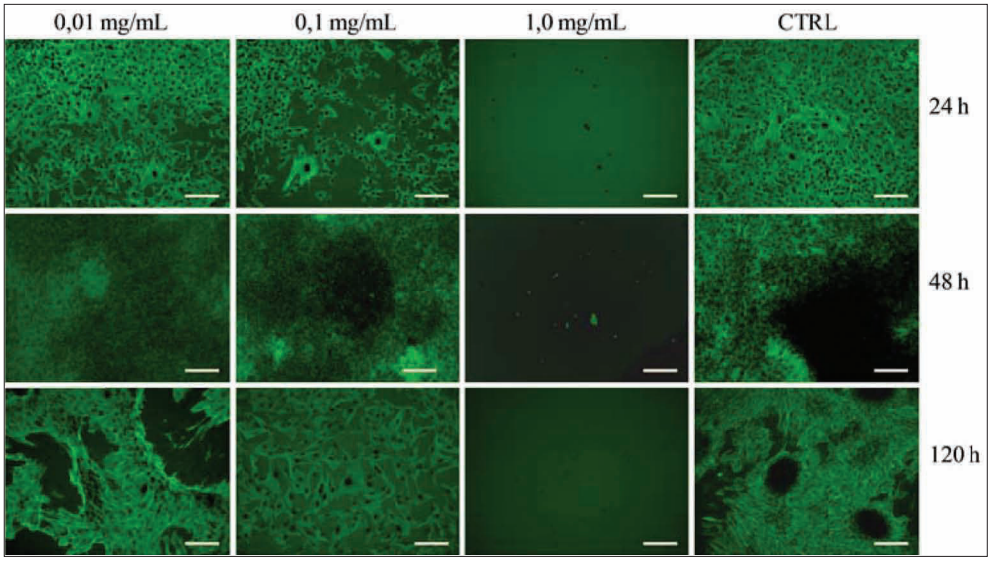
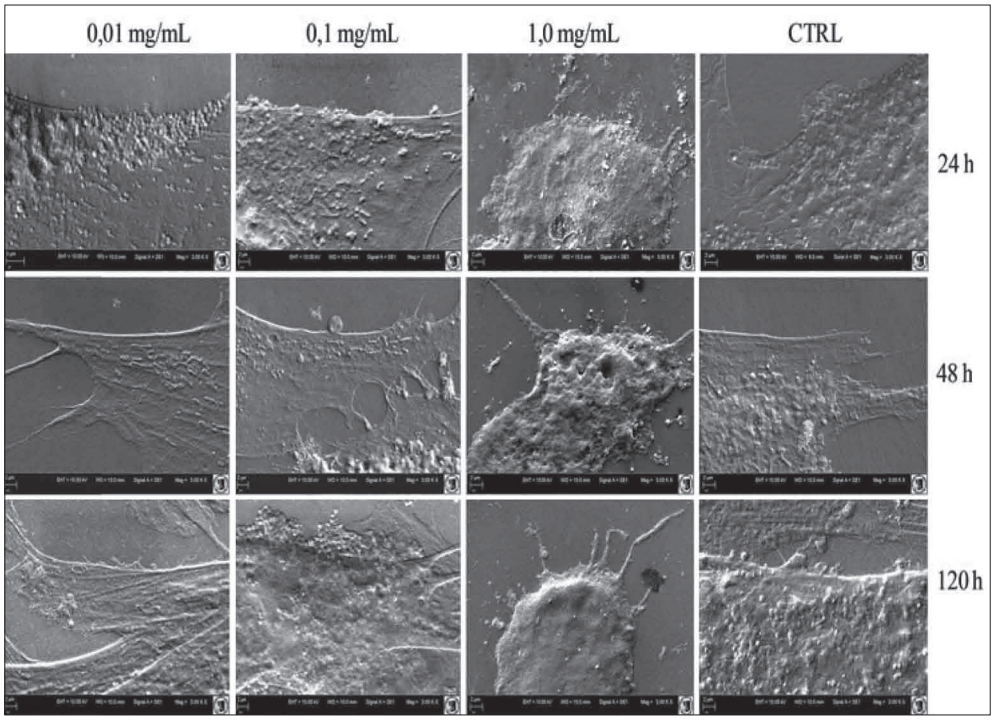


Fig 4. Morphological changes of equine AdMSCs' treated with different concentration of flunixin meglumine; Scale bar = 200 mm

Şekil 4. Değişik flunixin meglumine konsantrasyonları ile muamele edilen at AdMSC'lerindeki morfolojik değişimler; Ölçek birimi: 200 µm

Fig 5. Microphotographs of canine AdMSCs treated with flunixin meglumine; Scale bar = 2 mm.

Şekil 5. Flunixin meglumine ile muamele edilen köpek AdMSC'lerinin mikrofotoğrafları; Ölçek birimi: 2 µm



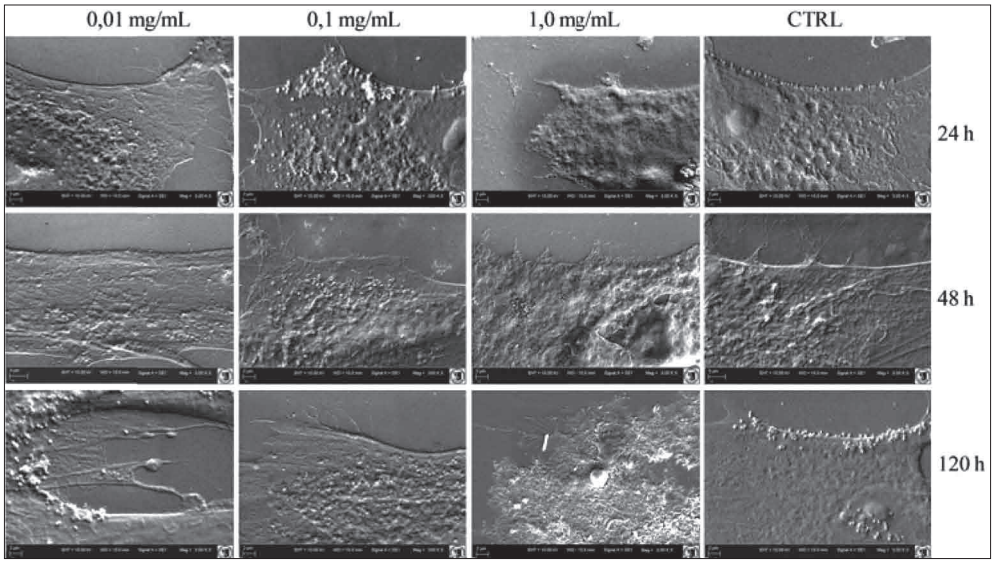


Fig 6. Microphotographs of equine AdMSCs treated with flunixin meglumine; Scale bar = 2 mm

Şekil 6. Flunixin meglumine ile muamele edilen at AdMSC'lerinin mikrofotoğrafları; Ölçek birimi: 2 µm

only the intracellular microvesicles location was noticed. A slight degree of extracellular distribution of mMVs in the canine culture was observed when 0.1 mg/ml of flunixin meglumine was used on the fifth day of the experiment.

DISCUSSION

In recent years, there has been an increased demand for effective anti-inflammatory and anti-pain therapy in veterinary medicine. This is mainly due to intensification of sport horse-riding activities, as well as hereditary diseases of many select dog breeds [19]. These factors lead to more frequent occurrence of diseases closely correlated with the locomotive and musculoskeletal systems. Due to advancement of diagnostic methods, disorders such as tendon and ligament injuries, rheumatoid arthritis (RA), osteoarthritis (OA) and hip joint dysplasia are more effectively diagnosed. As a consequence, treatment may be undertaken in the early stages of the disease. For many years, NSAIDs have been the one and only alternative for pain treatment [20]. Patients that manifest strong pain and inflammation issues require constant veterinary intervention. There are studies which demonstrate the positive effects of NSAIDs *in vivo,* especially when pain management is considered. However, there are reports indicating that prolonged NSAIDs usage causes many side effects [1,21-22]. This *status quo* forces the development of medical alternatives, which may lead to better anti-inflammatories and may even contribute to the re-creation of damaged tissue. Such alternatives entail regenerative medicine. This new branch of medicine is strongly developing, especially in the field of veterinary orthopedics. In the last decade, scientists devoted more attention to applied stem cells both in the case of dogs and horses.

Our previous studies [10,11] demonstrated the positive effects of stem cells application for tendon disorders and spar treatment in horses. These results were also confirmed by other research groups, which showed regenerative potential of adult stem cells in a dog's osteoarthritis treatment [23]. In the course of a clinical procedure, NSAIDs application is routinely applied, especially in the early stages of treatment [24]. Therefore, in this research we decided to investigate the influence of flunixin meglumine on the morphology and proliferation of canine and equine adipose-derived mesenchymal stem cells. Our findings showed a stimulating effect of 0.01 mg/ml of the investigated drug on canine stem cells proliferation, but only for the first 48 h of the experiment. In further stages, after 120 h, we observed a significant slowdown of CaAdMSCs proliferation rate when compared to the control culture.

Higher concentrations of flunixin meglumine (0.1 mg/ml) resulted in a lower proliferation rate but surprisingly, canine stem cells revealed typical morphology only when this concentration was applied. When 0.01 and 1.0 mg/ml of flunixin meglumine was investigated, untypical, clustered growing patterns were observed.

Comparing morphological features of Canine (Ca-) and Equine (Eq-) AdMSCs, the infulence of 0.1 mg/ml flunixin meglumine resulted in a properly maintained phenotype of cells. It proves that the use of flunixin meglumine yields similar results for both stem cell populations. However, when proliferation rates are considered, EqAdMSCs, in the presence of all investigated drug concentrations, revealed significant inhibition of their activity. This can be explained by different mechanisms of drug metabolism pathways of canine and equine stem cells. Our results partially correlate with Muller et al.[2] findings where positive effects of NSAIDs (in lower concentrations) in EqAdMSCs was observed. Those findings strongly correlate with the presence and localization of mMVs on the AdMSCs' surface. When cytophisiological activity of Ca and Eq AdMSCs is considered, the lowest investigated concentration of flunixin meglumine caused a beads-like distribution of

mMVs. The rest of the drug concentration applied in the experiment caused poor secretion and distribution of mMVs.

Taken together, we conclude that clinical application of flunixin meglumine combined with stem cells in canine treatment may have a positive effect, but only when 0.01 mg/ml concentration is used. These conclusions are opposite to the observations made for EqADMSc. On the basis of the obtained results, we suggest that flunixin meglumine may significantly inhibit AdMSCs activity; therefore combined therapy for equine treatment may lead to an unsatisfactory clinical effect.

However, even 0.01 mg/ml concentration of flunixin meglumine resulted in lower viability after 120 h of the canine culture when compared to the control.

Our results provide practical information for veterinary clinicians concerning combined therapy of AdMSCs and a widely used NSAIDs drug, flunixin meglumine. We showed that the applied drug is not inert for AdMSCs and even in low doses, affects the cells proliferation ratio. However, at concentration 0.01 mg/ml, no significant changes in cell morphology was observed. Based on the obtained results, we conclude that flunixin meglumine would be a more advantageous approach to canine treatment.

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