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Original article

The effect of metamizole and tolfenamic acid on canine and equine adipose-derived mesenchymal stem cells (ASCs) an *in vitro* research.

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Abstract

The influences of NSAIDs (Nonsteroidal Anti-inflammatory Drugs) – non-selective metamizole and selectively-acting tolfenamic acid were estimated on morphology, ultrastructure, and cytophysiological activity of canine (Ca) and equine (Eq) adipose-derived mesenchymal stem cells (ASCs). The lowest concentration of metamizole (0.01 mg/mL) stimulated the viability and cytophysiological activity of Ca ASCs and did not affect cell morphology. Stimulated cells possessed a proper, fibroblastic shape, with large, eccentrically located nuclei. Similar effects to those observed in Ca ASCs were found in Eq cells treated with both drugs. Cells cultivated with the intermediate (0.1 mg/mL) doses of NSAIDs displayed proper cell morphology, whereas cells cultivated in intermediate dose (0.1 mg/mL) became more flattened. The highest concentrations (1 mg/mL) of both drugs resulted in a cytotoxic effect in Ca and Eq ASCs. Based on these results, we conclude that stimulation of Ca and Eq ASCs with metamizole as well as Eq ASCs with tolfenamic acid can lead to positive effects only when the lowest drug concentrations are applied. This study indicates a different cellular response of canine and equine ASCs treated with metamizole and tolfenamic acid. The obtained data might be potentially useful in the study of functionalized veterinary biomaterials.

Key words: adipose-derived mesenchymal stem cells, non-steroidal anti-inflammatory drugs, cell morphology, canine, equine

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Introduction

Nowadays, musculoskeletal disorders, such as osteoarthritis (OA), are the most frequently occurring therapeutic problem in veterinary medicine. In equine medicine, it has been shown that approximately 60% of lameness is related to OA (Caron and Genovese 2003). This chronic degenerative disease is also one of the common causes of lameness in dogs. A survey conducted by the National Animal Health Monitoring Systems Program found that only in the USA, more than 20% of dogs are diagnosed with arthritis (Black et al. 2007). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly applied medications in orthopedic veterinary medicine. This group of drugs is widely used as a therapy of choice for mild pain treatment or as a concomitant therapy. Administration of NSAIDs is particularly effective and of great importance in the postoperative period (Luna et al. 2007). There is a great variety of anti--inflammatory drugs, which markedly differ in chemical composition, potency and toxicity, but all of them possess one common feature – they inhibit the catalytic activity of two distinct cyclooxygenase isozymes, i.e., COX-1 and COX-2, and thus the synthesis of inflammatory mediators, such as prostaglandins (PGs) and thromboxanes (TXs) (Crofford 1997, Simons et al. 2004, Crofford 2013). It is well known that these factors are responsible for inflammation, swelling, pain, and fever. Administration of NSAIDs leads to a reduction of histamine and/or bradykinin release, thereby suppressing the ongoing inflammatory response (Müller et al. 2011). Although many advantages of NSAIDs have been reported (e.g., non-addictive nature, possibility of prolonged administration, effective pain relief), their application is also associated with many side effects, such as gastric ulcers, nephrotoxicity, and digestive disorders (Simons et al. 2004, Crofford 2013). Therefore, modern medicine is continually looking for new healing solutions.

Regenerative medicine is one of the fastest growing fields of medicine, aimed at replacing damaged tissue with new tissue with complete functionality. Stem cell therapy appears to be a very promising approach due to the crucial properties of stem cells, such as their multi-potent state (ability to differentiate into multiple cell lineages), capacity for self-renewal, plasticity, and immunomodulatory features manifested by the reduction of inflammatory processes; therefore, their use in veterinary medicine is desirable (Gonzalez et al. 2009, Marycz et al. 2013). Autologus mesenchymal stem cells (MSC) injections were performed for example in equine degenerative joint disease (DJD) or canine osteoarthritis (Black et al. 2007, Qui et al. 2012, Marycz et al. 2012ab). The regenerative potential of stem cells is probably associated with their paracrine action, which in turn is correlated with the synthesis of microvesicles and their release to the intracellular space. It is well documented that mesenchymal microvesicles (mMVs) shed by adult MSCs are rich in a broad spectrum of cytokines and growth factors, for instance, VEGF – Vascular Endothelial Growth Factor, HGF – Fibroblast Growth Factor, IGF-I – Insulin-like Growth Factor 1, MCP-1 – Monocyte Chemo attractant Protein-1 or BMPs – Bone Morphogenetic Proteins. These active substances exert their biological effects on cells within their close vicinity; namely, they can suppress local inflammation and immune response or stimulate proliferation and differentiation of cells. Since mMVs can be taken up by the neighboring cells, they are a significant instrument of cell-to-cell communication and contribute to a proper cellular response (Biancone et al. 2012). Therefore, evaluation of localization, size, and density of mMVs seems to be crucial in the assessment of ASCs potential in terms of the regenerative process. Moreover, the presence of cytonemes and tunneling nanotubules (thin membrane bridges), which play a role in cell-to-cell signaling and intercellular transport, indicates cytophysiological activity of MSCs and correlates well with the natural growth rate of these cells (Lou et al. 2012).

Stem cell therapies, together with the application of various types of NSAIDs, are frequently used in the treatment of patients with orthopedic diseases (Marycz et al. 2012ab). It is reasonable to study the effect of NSAIDs on mesenchymal stem cells (MSCs), as the combined therapies might give promising outcomes in clinical practice, resulting in an effective reduction of pain and inflammation. The aim of the present study was to evaluate the morphology and cytophysiological activity of canine and equine adipose-derived mesenchymal stem cells cultured with two commonly used NSAIDs, i.e., metamizole and tolfenamic acid. The majority of adverse effects caused by NSAIDs (e.g., gastrointestinal complications, prolonged bleeding time, and kidney disease) results from the inhibition of COX-1 activity (Urban 2000) and stem cell therapy, combined with administration of drugs inhibiting COX-2 activity, seems to be a promising solution for DJD treatment. In this work, we evaluated whether non-selective or selective NSAID may be more beneficial in canine and equine OA treatment. In order to investigate the effect of these drugs on stem cell signaling, we assessed the localization and density of microvesicles, as well as the presence of cytonemes and tunneling nanotubules in cultured stem cells.

Materials and Methods

Ethical Approval

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

Isolation of ASCs

Mesenchymal stem cells were isolated from subcutaneous fat tissue, under local anesthesia, from a male horse and a female dog. The owners of the animals provided proper consent for the procedure. Both animals were five years old without signs of disease. All stages of the experiment were performed as described previously (Grzesiak et al. 2011 ab). Briefly, fat tissue samples (2 g) were washed extensively with sterile Hank's Balanced Salt Solution (HBSS, 55021C, Sigma- Aldrich, Poland) containing 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich, Poland) to remove contaminating debris and red blood cells. Tissue fragments were finely minced and digested with collagenase, at a concentration of 1 mg per ml of culture medium (C9891, Sigma-Aldrich, Poland). In order to facilitate enzyme action, samples were placed in a cell culture incubator (temperature 37°C, 5% CO_2) for 30 minutes. The cells were then centrifuged at 1200xg for ten minutes. After centrifugation, the supernatants were collected and discarded, while pellets of the stromal-vascular adipose fraction (SVF) containing MSC precursors were re-suspended in a culture medium.

Propagation of cells

Cells were cultured at 37°C in a 95% air and 5% CO2 atmosphere. The culture medium was changed every two days, and non-adherent cells and debris were removed from the culture flask. For the primary culture, Dulbecco's Modified Eagle's Medium (DMEM) and Nutrient mixture F-12 Ham (51448C, Sigma-Aldrich, Poland) was supplemented with 15% fetal bovine serum (FBS, 12003C, Sigma-Aldrich, Poland) and 1% of antibiotic-antimycotic solution. The secondary cultures were maintained in DMEM (D5796, Sigma Aldrich, Poland), containing 4500 mg/L of glucose with an addition of 10% FBS and 1% of antibiotic-antimycotic solution

Non-steroid anti-inflammatory drugs (NSAIDs)

Metamizole (40 mg/ml. Biovetalgin, Biowet Drwalew S.A, Poland) and Tolfenamid acid solution (40 mg/ml; Vetoquinol Biowet Sp. z o.o., Poland) were tested *in vitro* as additives to the culture medium at the following concentrations: 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL.

Evaluation of NSAIDs effect on ASCs

In vitro evaluation of NSAIDs effects on canine and equine ASCs was performed in 24- well plates. The initial concentration of *inoculum* was $2x10^4$ cells in 0.5 ml medium per well. The first dosage of the investigated drugs was added to the medium after six hours, when adhesion and spreading of cells on the plate surface were observed. Non-treated cells served as a control for comparison with the test culture. All samples were prepared in duplicate. During cell propagation, the medium was changed at 48-hour intervals, and cultures were split every 2-3 days. The morphology and viability of the cells were evaluated after 24, 72, and 120 hours.

Cytotoxicity of ASCs and the rate of cell proliferation

The degree of cytotoxicity induced by the drugs was assessed by evaluating the metabolic activity of living cells using a resazurin-resorufin system (Alamar Blue, Invitrogen, USA) as described previously (Krzak-Ros et al. 2013, Marycz et al. 2013, 2014). This *in vitro* cytotoxicity kit is used as an oxidation-reduction indicator in mammalian cell viability assay. To perform the test, the culture media were removed and replaced with a media containing 10% of the dye. Cells were placed in a CO_2 incubator for two hours, and then the supernatants were collected and transferred into the 96-well microplate reader (Spectrostar Nano, BMG Labtech). The absorbance of the supernatants was measured spectrophotometrically, at a wavelength of 600 nm for resazurin, and 690 nm as a reference wavelength. Each test included a blank containing a complete medium without cells. To evaluate the proliferation rate, a standard curve of a range of cell concentrations ($2x10^4$, $4x10^4$, $8x10^4$ and $16x10^4$) was calculated with absorbance directly proportional to the number of cells. Significant differences between particular proliferation factors were estimated with one-way analysis of variance (ANOVA) and Dunn's multiple range tests (STATISTICA, version 7.1, StatSoft, Inc. Data Analysis Software Sys-

tem). Differences with a probability of $P<0.05$ were considered significant.

Morphology of ASCs

Morphology of the investigated cells was evaluated under an inverted phase contrast microscope and epifluorescent microscope (Zeiss, Axio Observer A.1). To assess the nuclei morphology and structure of the actin filaments, fluorescent staining was performed. Cell nuclei were visualized by staining with diamidino-2-phenylindole (DAPI; blue), whereas actin filaments were visualized using fluorochrome-conjugated phalloidin (red).

MVs evaluation

To evaluate the cellular physiological activity of ASCs, the density of mMVs was analyzed in the investigated cells using a scanning electron microscope (SEM, EVO, LS-15) by means of the methods described previously (Kania 2009, Kalinski et al. 2012, Witek-Krowiak 2013). The number of MVs was analyzed with AxioVision image processing software (Zeiss®) on seven randomly chosen fields of previously captured SEM images. One-way ANOVA and Fisher's multiple range tests were applied to calculate statistical significance of mean differences, (STATISTICA, version 7.1, StatSoft, Inc. Data Analysis Software System). Differences with the probability of $P<0.05$ were considered significant.

Results

Cell culture growth and morphology evaluation

The present data demonstrated that metamizole and tolfenamic acid affected the morphology of both canine and equine ASCs in dose-dependent manner. In canine and equine ASCs cultured in the presence of the lowest concentration (0.001 mg/mL) of both drugs, proper cell morphology throughout the entire experimental period was observed as compared to the controls. Cultured cells were heterogeneous with characteristic spindle- and flatten- shape, and prominent, centrally located nuclei. However, an intermediate dose (0.1 mg/mL) of metamizole led to the development of a flattened morphotype in both ASCs in comparison to the controls. Moreover, in canine ASCs, the presence of a substantial heterogenic cell population was observed with occasional fibroblast-like cells. Intermediate doses of tolfenamic acid triggered a cytotoxic effect in canine ASCs, while administration of metamizole in equine cultures caused a cylindrical, flattened cell shape with a well-developed cytoskeleton and centrally located nuclei. In contrast to the control culture, where a typical dense fibroblast-like monolayer was observed, intermediate concentrations of tolfenamic acid caused a thinner cellular film. In turn, cells treated with higher concentrations of both NSAIDs had lower viability, indicating a strong negative impact of these compounds on cell survival. The highest concentrations (1 mg/ml) of both drugs investigated resulted in cytotoxicity of all the cultured cells. However, abnormal cell morphotype, after tolfenamic acid administration, was more pronounced in canine cultures. On the last day of incubation, no viable cells were present in the culture. These cells were present in the first two days of incubation and were not detected on the last day of the study.

Evaluation of cell morphology with scanning electron microscopy (SEM)

The SEM technique allowed for a more precise visualization of the ultra-structure of examined cells and confirmed the results obtained using the inverted light microscope. Individual stem cell morphology could be described as bi- three- or multi-angled, with a relatively high nuclear-cytoplasmic ratio. The cells were distinguished by a spindle shape and large nuclei with prominent nucleoli. Cells with abundant amounts of mMVs (edge- and surface-placed) had clear cytonemes and tunneling nanotubules (Figs. 1,2).

In the cultures of canine and equine ASCs, the highest dosage (1 mg/mL) of the study drugs led to cell death during the entire course of experiment, and only apoptotic and/or necrotic cells were found in the samples. In turn, the intermediate concentrations (0.1 mg/mL) of NSAIDs resulted in significant changes in the organization of the cytoplasm, in which sponge- or foame-like structures were found. Moreover, a low number of cell-to-cell connections were particularly apparent in the canine ASCs (Figs. 1,2). There were no significant changes in cell morphology when the lowest concentration of NSAIDs was added to the media.

Evaluation of microvesicles

Application of the test drugs resulted in a noticeable increase in the density of mMVs in ASCs. This observation was confirmed by morphometric evaluation of cells, as well as by statistical analysis. The

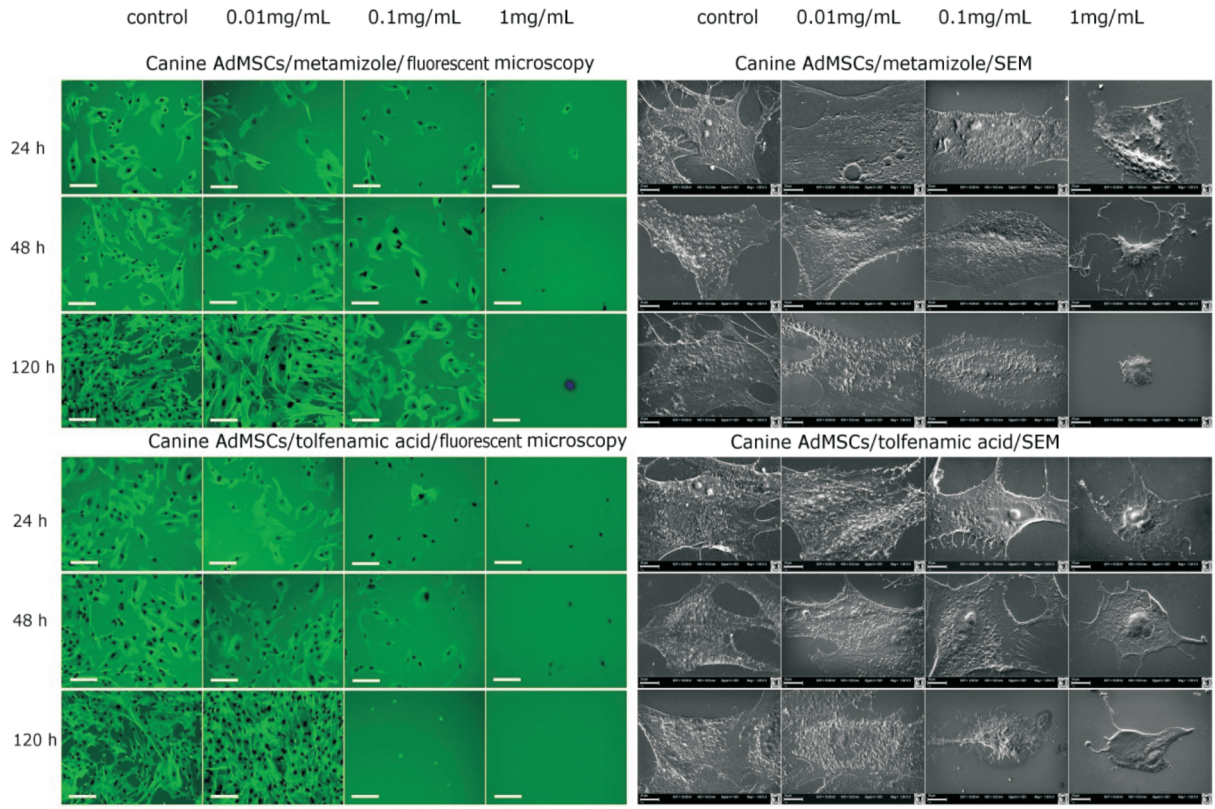

Fig. 1. Morphological changes of canine ASCs treated with different concentrations of metamizole and tolfenamic acid after 24, 48 and 120 hours of incubation. Images taken using inverted fluorescent microscope (on the left) and scanning electron microscope (on the right). Scale bars: 150 µm for inverted fluorescent microscope and 10 µm for SEM.

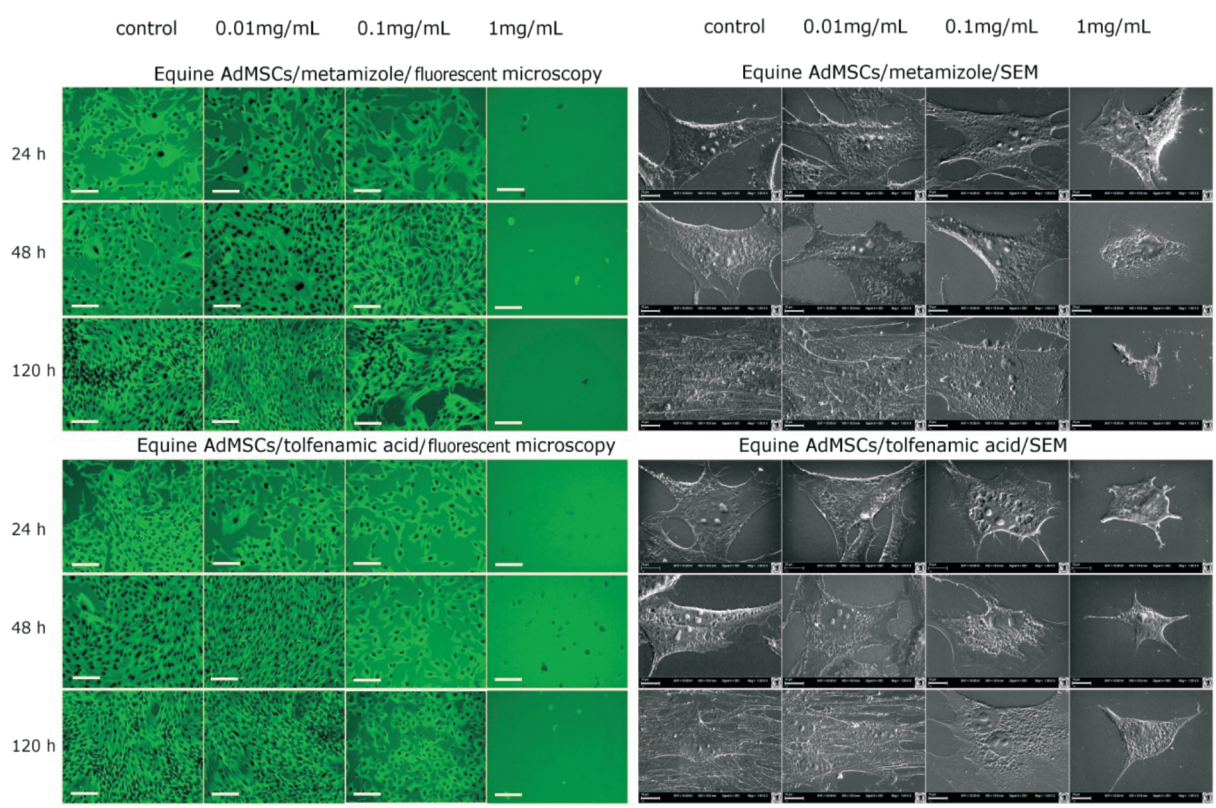

Fig. 2. Morphological changes of equine ASCs treated with different concentrations of metamizole and tolfenamic acid after 24, 48 and 120 hours of incubation. Images taken using inverted fluorescent microscope (on the left) and scanning electron microscope (on the right). Scale bars: 150 µm for inverted microscope and 10 µm for SEM.

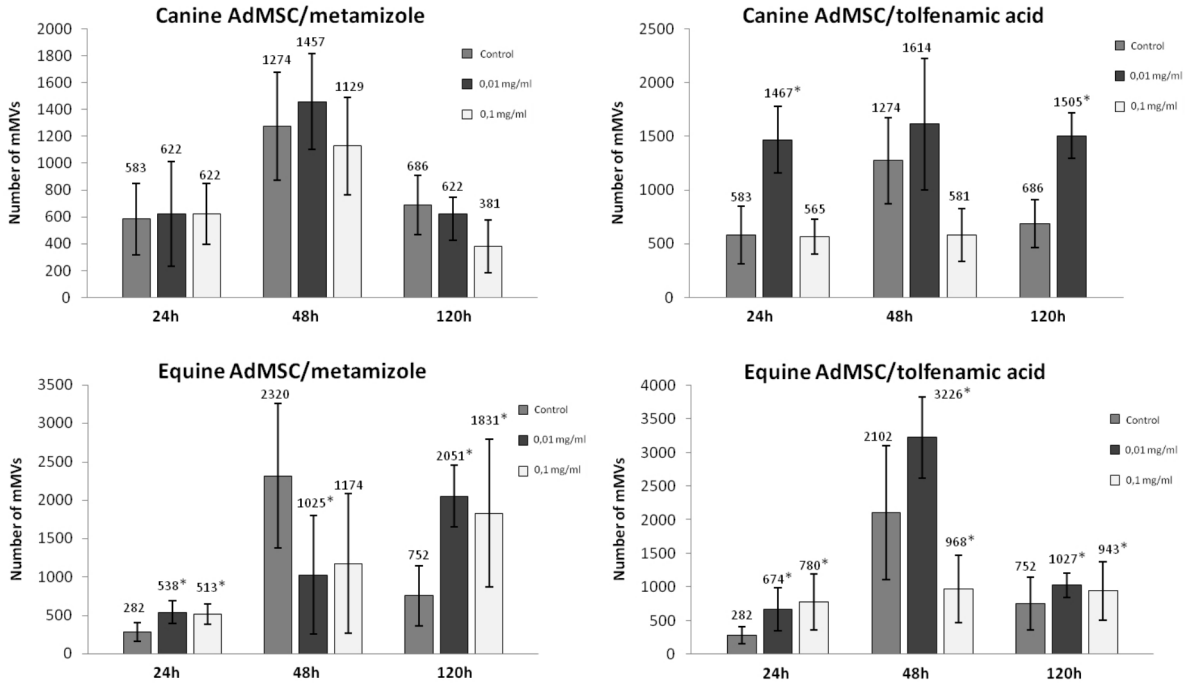

Fig. 3. Mean values ±SD (n=7) of effects of NSAIDs on the number of mesenchymal microvesicles in canine and equine ASCs cultures after 24, 48 and 120 hours of incubation. Significant differences (P<0.05) between drug-treated cells and control cultures are marked with an asterisk. ND refers to not detectable number of MVs.

Table 1. Proliferation rate of canine and equine ASCs treated with a defined concentration of metamizole and tolfenamic acid after 24, 48 and 120 hours of incubation. The factor is expressed as an arbitrary unit (mean values ±SD, n=7). PF value for the control cultures was established as equal to one.

Canine ASCs												
Culture time	metamizole						tolfenamic acid					
	0.01 mg/mL		0.1 mg/mL		1 mg/mL		0.01 mg/mL		0.1 mg/mL		1 mg/mL	
	PF	±SD	PF	±SD	PF	±SD	PF	±SD	PF	±SD	PF	±SD
24 h	1.52	0.35	1.34	0.39	0.91	0.31	1.63	0.64	0.78	0.29	ND	ND
48 h	1.10	0.14	1.08	0.07	0.91	0.14	0.92	0.17	0.54	0.09	0.06	0.16
120 h	0.49	0.04	0.17	0.04	0.06	0.03	0.94	0.04	0.04	0.02	ND	ND
Equine ASCs												
Culture time	metamizole						tolfenamic acid					
	0.01 mg/mL		0.1 mg/mL		1 mg/mL		0.01 mg/mL		0.1 mg/mL		1 mg/mL	
	PF	±SD	PF	±SD	PF	±SD	PF	±SD	PF	±SD	PF	±SD
24 h	1.17	0.18	0.90	0.11	0.61	0.16	1.40	0.24	0.86	0.10	ND	ND
48 h	1.16	0.06	0.95	0.08	0.58	0.09	1.10	0.08	0.71	0.04	0.35	0.08
120 h	1.11	0.02	0.87	0.05	0.14	0.03	1.04	0.04	0.24	0.02	0.03	0.03

Abbreviations: PF – proliferation factor, SD – standard deviation, ND – not detectable proliferation activity of cells.

number of mMVs in canine cells incubated with tolfenamic acid (0.01 mg/mL) and in equine cells treated with both drugs (0.01 mg/mL) was significantly higher (p<0.05), as compared to the control. However, in canine cultures incubated with tolfenamic acid (0.1 mg/mL), and equine culture with both types of NSAIDs (0.1 mg/mL each), the number of mMVs was considerably lower in comparison to the number of mMVs in the cells that were exposed to the lowest concentration of the test drugs (0.01 mg/mL; Fig. 3). Substantial differences in the distribution of mMVs were observed between equine and canine cells. After

administration of the test drugs (0.01 mg/mL dose of each drug) to the cultures, in equine cells, the vast majority of bead-like arrangements of mMVs was observed at the edges of the cell; whereas in the canine cells, they were equally distributed over the entire surface of the cell when compared to the control (Figs. 1,2).

Proliferation rate

Proliferation factor (PF, expressed as an arbitrary unit) of canine and equine ASCs was dependent on the concentration of medicine used in the study. Both drugs investigated, at a concentration of 0.01 mg/mL, caused an increase ($P<0.05$) of the equine stem cell proliferation rate throughout the entire period of the study when compared to the controls. In the case of canine cells, metamizole and tolfenamic acid enhanced ($P<0.05$) the PF, but only within 24 hours of incubation. Intermediate (0.1 mg/mL) doses of NSAIDs exerted no or only a slightly adverse effect on the proliferation rate of ASCs in canine and equine cells, whereas the highest (1 mg/mL) doses of drugs resulted in a strong toxic effect in all cases (Table 1).

Discussion

In veterinary medicine, musculoskeletal disorders affecting dogs and horses are a commonly occurring therapeutic problem (Caron and Genovese 2003, Black et al. 2007). For many years, NSAIDs have been the only alternative for the management of pain. However, many reports pointed out harmful effects of NSAIDs, manifested in various side effects during prolonged administration of these drugs (Crofford 2013). Therefore, the development of alternative strong anti-inflammatory therapies, leading to the repair of damaged tissue, is strongly desired. One of the most promising future techniques in the medical arsenal, especially in the area of veterinary orthopedics, is regenerative medicine. There is a growing body of evidence that MSCs are useful in tissue engineering, especially in canine and equine veterinary medicine (Qui et al. 2012, Marycz et al. 2012a,b). Combinations of these cells, particularly ASCs, with specific types of NSAIDs, seem to be the most effective solution for the current orthopedic treatment of patients.

In the present study, the effect of two routinely used NSAIDs, a non-selective metamizole and a selectively-acting tolfenamic acid have been investigated on canine and equine ASCs *in vitro,* with regard to morphological, ultrastructural and cytophysiological characteristics. The present data demonstrated that metamizole and tolfenamic acid affected the morphology of the cells in a dose-dependent manner. In particular, the cells treated with the highest concentrations (1 mg/mL) of both investigated drugs resulted in high cytotoxicity of all the cultured cells. However, an abnormal cell morphotype, after tolfenamic administration, was more pronounced in canine cultures. Our findings are in line with other studies, where the highest concentrations of NSAIDs induced cell death (Ho et al. 1999, Chang et al. 2005, 2006, 2007, 2009). After the addition of selective NSAIDs (celecoxib) to the culture, apoptotic and necrotic changes were discovered in the morphology of osteoblasts and bone mesenchymal stem cells derived from humans (Chang et al. 2009) and rodents (Chang et al. 2007), respectively. Toxic effects of NSAIDs were also indicated in calvarial osteoblasts (Chang et al. 2005) and epiphyseal-articular chondrocytes in rats (Chang et al. 2006). As we demonstrate here, the cytotoxic effect of tolfenamic acid on canine ASCs may result from the higher sensitivity of these cells to the test drug. It is possible that canine cells might be more resistant to the adverse effects of selective NSAIDs than equine cells. Although the mechanism of these differences is unclear, we speculate that the higher concentration of tolfenamic acid may lead to changes in the expression level of pro-apoptotic and anti-apoptotic genes. This supposition is based on the study performed by Chang (2009) where the administration of celecoxib was associated with an enhancement of the expression level of the Bak gene (involved in apoptotic pathway), as well as with a decline in the expression of the Bcl-XL gene (involved in survival/death pathway), induced by dexamethasone (corticosteroid). It is worth noting that in equine cells cultured with the lowest doses of the study drugs, as well as in the control cultures, a small number of large, polinucleated cells, so-called "giant cells", were noticed. This type of cells might represent a macrophage line with phagocytic properties, as previously reported (Grzesiak et al. 2011). Giant cells are formed due to nuclear division without cytokinesis and are prone to changes in various pathologies, such as fibrosis, cancer, aging, and the foreign body response (Holt and Grainger 2011). A larger population of giant cells observed in our study of equine cultures cultivated with metamizole, compared to cultures exposed to tolfenamic acid, may indicate increased activity of COX-2, induced by an intensified pro-inflammatory immune response. This assumption is confirmed by the fact that COX-2 is specifically up-regulated by inflammatory stimuli and becomes abundant in activated macrophages and other cells at the sites of inflammation (Cipollone et al. 2001).

Our data showed that the dynamics of the culture growth was changing throughout the entire experi-

mental period and was correlated with the dose of study medications. It was established that the lowest (0.01 mg/mL) and intermediate (0.1 mg/mL) concentrations of metamizole positively influenced canine mesenchymal stem cells, stimulating higher proliferation within the first 48 hours of the experiment. In the later stages of the culture (120 hours), a significant decrease of canine ASC proliferation has been observed as compared to the controls. Similarly, a positive effect on the proliferation rate of equine ASCs- until the fifth day of the experiment- was observed for the lowest concentration of both NSAIDs. These results correlated well with the findings of Muller et al. (2011) where a positive impact was found, with the lowest concentration of non-selective NSAIDs (celecoxib, meloxicam, flunixin), on the proliferation factor of equine ASCs. Furthermore, Marycz et al. (2013), showed a stimulating effect with the lowest concentration of flunixin on the proliferation rate of canine stem cells during the first 48 hours of the experiment. When the intermediate doses of both metamizole and tolfenamic acid were evaluated in the horse cells, a significant inhibition of cell activity throughout the entire experimental period was observed. Moreover, in comparison to the controls, the highest concentration (1 mg/mL) of both metamizole and tolfenamic acid caused a significant decrease of proliferation in canine and equine stem cells. A strong cytotoxic effect of tolfenamic acid on canine and equine stem cells was recorded in the first and last day of the culture, when cell death was detected. This finding is partially consistent with the previous study performed by Wang et al. (2006), where the highest concentrations of aspirin added to the medium were effective in inhibiting the growth of MSCs, without concomitant increase in cell death. It can be assumed that the different values of proliferation observed in our study in ASCs isolated from various species may be due to the different mechanisms of drug metabolism pathways in canine and equine stem cells.

We have shown that the proliferation factor of cells studied was strongly correlated with the density and localization of mMVs on the surface of ASCs. Compared to the controls, cytophysiological activity of ASCs, expressed by the number of mMVs, was enhanced in canine cells when the lowest concentration of tolfenamic acid was applied. A similar phenomenon was observed in equine cells treated with the lowest concentration of both NSAIDs throughout the entire experimental period. Moreover, intermediate doses of metamizole and tolfenic acid led to an increased density of microvesicles in equine cells throughout entire experiment when compared to the controls. These results are in line with the previous studies by Marycz et al. (2013), where increased density of mMVs in canine and equine ASCs was observed after administration of the lowest and intermediate concentrations of flunixin and persisted until the last day of the experiment. In addition, when the distribution of mMVs is considered in canine and equine cultures treated with both drugs, intra- and extracellular microvesicles were found. It was shown that an increase in the concentration of the drug was accompanied by an elevated number of secretory vesicles. In canine and equine ASCs, cultured with metamizole and tolfenamic acid, a certain number of shedding vesicles was present within the larger vesicles in the cytoplasm. We suppose that these structures represent a fraction of the MVs that were taken up by the target cells through endocytosis (Cocucci et al. 2009). It is worth noting that cell-to-cell communication associated with the shedding of mMVs was observed. Increased activity of ASCs, following the application of the lowest doses of the study drugs, was manifested not only by the high proliferation factor and the number of MVs produced, but also by the formation of specific cellular structures, i.e., cytonemes and tunneling nanotubes. Based on the available data, we assume that the presence of microvesicles at the wound site could reflect a pro-regenerative potential of MSCs. It is well known that MVs are rich in numerous bio-active factors and play a crucial role in paracrine and autocrine cell signaling (Ratajczak 2011). We presume that a thorough analysis of the impact of NSAID drugs on MSCs biology will bring new potentially therapeutic strategies. Furthermore, a detailed analysis of the cytophysiological assessment of stem cells allows veterinarians to select cells with the highest regenerative capability, which is a key factor in the later stages of therapy.

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