EVALUATION OF THE *IN VITRO* EFFECT OF DIFFERENT CONCENTRATIONS OF FLUNIXIN ON LEUKOCYTES OBTAINED FROM CATTLE OF VARIOUS AGES

Andrzej Wernicki[*], *Renata Urban-Chmiel, Andrzej Puchalski, Marta Dec, Diana Stęgierska, Anna Dudzic*

SubDepartment of Veterinary Preventive and Avian Diseases, Institute of Biological Bases of Animal Diseases
University of Life Sciences Lublin
Akademicka 12, 20-033 Lublin, Poland

*Corresponding author: dr. hab. Renata Urban-Chmiel-mail: renata.urban@up.lublin.pl
Phone: +48 81 445 60 32, Fax: +48 81 445 60 32

Abstract. In view of the varied mechanisms of the of non-steroidal anti-inflammatory drugs, the aim of the study was to evaluate the *in vitro* effect of different concentrations of flunixin meglumine on viability, apoptosis, oxidative stress and chemotaxis of leukocytes isolated from cattle of various ages.

The material for the study consisted of leukocytes obtained from blood collected into EDTA tubes from the external jugular vein of Holstein-Friesian cattle aged 1 week to 2.5 years. The cells were cultured in RPMI 1640 medium with 7 % FBS and flunixin meglumine at a concentration of 33 or 15.6µg/ml.

Determinations were made of the following properties in the leukocytes: viability percentage by the trypan blue test and by flow cytometry, chemotaxis in a 48-well Boyden chamber, metabolic activity by NBT, and apoptosis.

The results obtained confirmed an insignificant negative *in vitro* effect of flunixin on the total viability of bovine leukocytes and the total viability of lymphocytes. A significant decrease in viability among the monocytes and neutrophils in calves at different ages, an inhibitory effect on chemotaxis and a significant effect on induction of apoptosis.

Keywords: flunixin meglumine, bovine leukocytes, apoptosis, chemotaxis

Introduction. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used medications, including for treatment of respiratory diseases in humans and animals. Flunixin, ketoprofen and carprofen have been used to reduce the disease process in cattle (Lockwood et al., 2003). Flunixin injections have proven particularly effective in treating infections induced by the PI-3 virus in combination with bacteria of the genus *Pasteurella* sp. (Selman et al., 1984). Combined use of antibiotic therapy and non-steroidal anti-inflammatory drugs significantly reduces the intensity of the disease process and substantially decreases the Bovine Respiratory Disease Complex (BRDC) mortality rate in calves.

The mechanism of action of NSAIDs mainly involves inhibition of the activity of cyclooxygenase (COX), which participates in metabolism of arachidonic acid and other unsaturated fatty acids (e.g. dihomo-γ-linolenic and eicosapentaenoic acids), so the efficiency of prostaglandins and lipooxegenase activity is suppressed. In addition to their analgesic effect, linked to suppression of COX-2, effects associated with digestive, respiratory, circulatory and urinary disturbances are obtained as well (Cuniberti et al., 2012; Paccani et al., 2005). The apoptotic activity of NSAIDs is associated with their ability to stimulate expression of genes responsible for apoptosis while at the same time blocking pathways controlling lymphocyte proliferation and viability (Paccani et al., 2003).

Flunixin occurring in the form of meglumine is a nicotinic acid derivative (1-Deoxy-1-(methylamino)-D-glucitol2-[2-methyl-3-(perfluoromethyl)anilino] nicotinate) and is included among non-selective cyclooxygenase inhibitors. It exhibits analgesic, anti-inflammatory and antipyretic activity. It reduces cell damage caused by bacterial toxins, counteracts lactic acidosis, and decreases tissue hypoxia.

The use of NSAIDs in combination with antibiotics to treat animals with respiratory infections significantly improves the effectiveness of treatment. The common use of NSAIDs and their accumulation in tissues may lead to disturbances in immune cell functioning, particularly when administered in amounts exceeding the doses recommended for a given species of animal. For example, flunixin and dexamethasone dose-dependently inhibit lymphocyte proliferation, but do not significantly reduce mRNA expression (Maeda et al., 2011). Flunixin meglumine may induce cytotoxic effects on lymphocyte cultures in mice, with the progression of these toxic changes dependent on the dose of flunixin (Aydin and Üstüner, 2009). In human polymorphonuclear leukocytes flunixin inhibits synthesis of leukotriene B$_4$ (LTB4) and influences migration of leukocytes (Kankaanranta et al., 1993). In view of the diverse mechanisms associated with the harmful effects of non-steroidal anti-inflammatory drugs on leukocytes during bovine respiratory syndrome, the aim of the study was to evaluate the *in vitro* effect of different concentrations of flunixin on viability, apoptosis, oxidative stress and chemotaxis of leukocytes obtained from cattle of various ages.

Material and methods

Blood samples preparation. The material for the study consisted of leukocytes obtained from whole blood collected into EDTA tubes from the external jugular vein of Holstein-Friesian cattle aged 1 week to 1 month (group1), group 2 (>1≤5 months), group 3 (above 5 months to 9 months) and group 4 (above 1year to 2.5 years during routine veterinary examinations. A total of

48 blood samples were taken (n=12 for each group of calves). The samples were transported for analysis at a temperature of 6–8° C within 2 hours after collection.

Isolation of cells from whole blood was carried out by density gradient separation using Histopaque 1083 (Sigma-Aldrich, Ge) according to Halliday et al. (2005). The cell suspension obtained, with a density of $5x10^6$/ml, was suspended in RPMI 1640 medium (Sigma-Aldrich, R5886, PL) with 7 % FBS (foetal bovine serum), penicillin G (100 U/ml) and streptomycin (100 μg/ml).

The total viability of the cells was evaluated directly after isolation determined by the trypan blue test (Sigma-Aldrich, PL) according to Sikora (1996). The viability percentage following 24–hour incubation in RPMI 1640 with flunixin meglumine was measured by flow cytometry of cells stained with propidium iodide (PI, Sigma-Aldrich, PL) in a flow cytometer (Epics XL Beckman-Coulter, Comesa CH-Werfen Company, USA) at 488nm (van Oostveldt Et al., 1999).

The cell cultures were carried out in 24-well plates (NUNC, Ge) in RPMI 1640 medium with 7 % FBS. A cell suspension at a concentration of $2.5x10^6$/ml was added in the amount of 500μl to each well containing 500μl RPMI 1640. Then flunixin at concentrations of 33μg/ml and 15.6μg/ml was added to each well. The control consisted of a leukocyte culture in RPMI 1640 medium. The plate was incubated for 24 h at 37°C in an atmosphere with 5 % CO_2 (Aydin and Üstüner, 2009; Kankaanranta et al., 1993).

Chemotaxis. The chemotactic activity of the neutrophils was determined using a 48-well Boyden chamber (R&D Systems, USA), with a nitrocellulose membrane 3 μm in diameter. The number of migrating cells was determined in an optical microscope (Olympus, JP) at objective 40x magnification. The chemotactic activity (%) of the cells exposed to the two concentrations of flunixin was determined according to a formula given by Alves et al. (1996) and presented in percentages.

NBT assay. The metabolic activity of the leukocytes was determined by nitro blue tetrazolium reduction (NBT) according to Pick (1986). A suspension of the 24-hour leukocyte culture in the amount of 100μl was dispensed into each well of a 96-well plate. The plate was incubated for 10 min. at 37°C, and then 100μl of NBT solution (1 mg/ml) suspended in phenol red free HBSS (Hanks' balanced solution, Sigma-Aldrich, PL) and 100μl of iodoacetamide were added to each well. Following 90-minute incubation at 37°C in an atmosphere with 5 % CO_2, absorbance was read with a 680 reader (BioRad, USA) at 550nm.

Nitric oxide assay. Content of NO ions released from the cells following incubation with flunixin meglumine was determined in a Griess reaction according to Misko et al. (1993).

Flow cytometric apoptosis. Apoptotic cells were identified using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™) according to the procedure recommended by the producer. Cytometric analysis was performed at 488 nm.

Statistical analysis was performed using Statistica 10.0 software (Statsoft, USA). The results were analysed using two-factors analysis of variance parametric and nonparametric in case of NBT assay descriptive statistics, median, range at a significance level of P≤0.05. The homogeneity of the variance for each evaluated parameter has been confirmed by Brown–Forsythe test. The normal distribution has been estimated by Shapiro-Wilk's test. Correlation of the results was analysed using Pearson's coefficient.
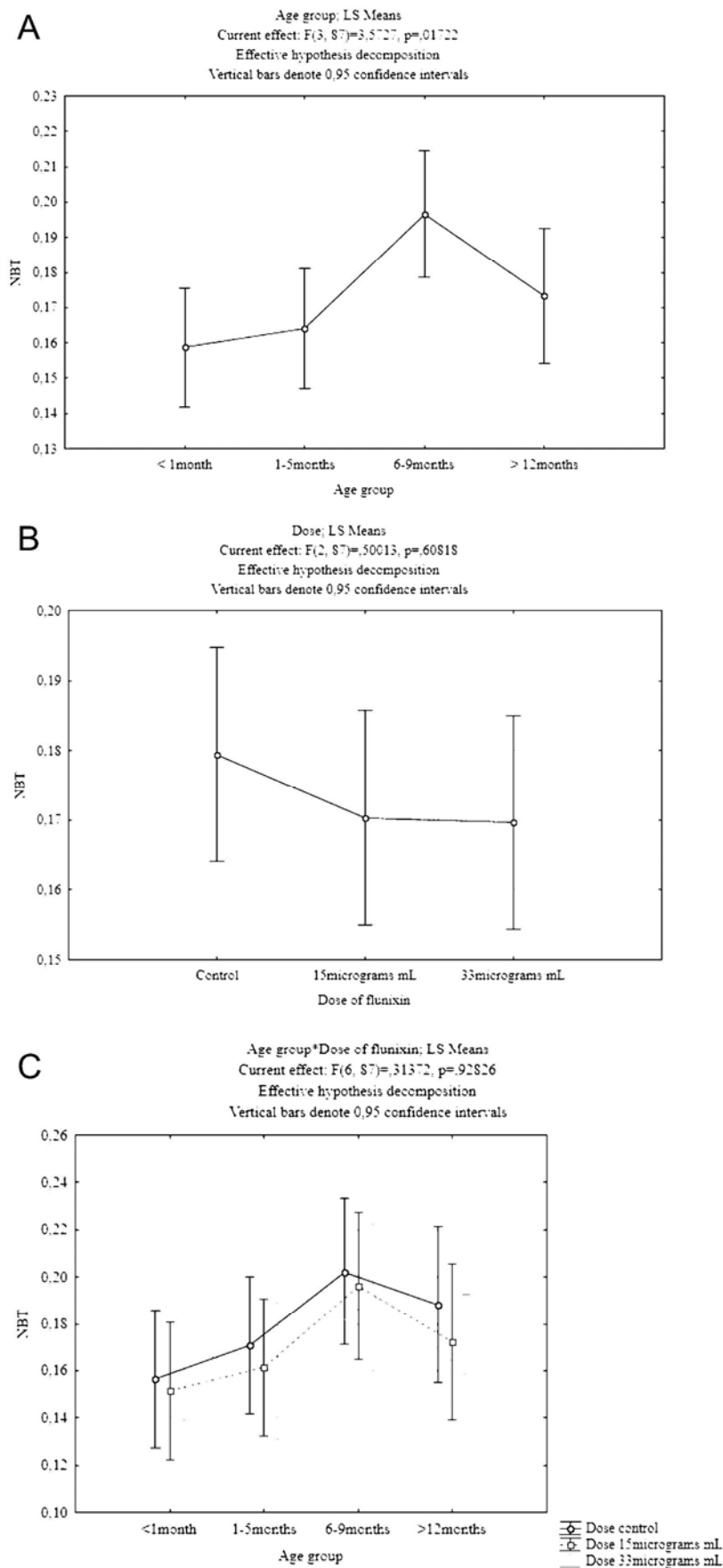
Results

A slight negative effect of flunixin on the total viability of leukocytes obtained from cattle of various ages was observed in *in vitro* conditions. The flunixin did not significantly affect the total viability of leukocytes, estimated by flow cytometry, which remained at of level 95 % in all age groups. A statistically significant (P≤0.05) decrease in viability was observed only in the case of monocytes in the group of calves aged >1≤ 5 (gr. 2) months and among neutrophils obtained from cattle aged 6-9 months. In the case of cattle aged 6-9 months, a significant (P≤0.05) reduction in monocytes viability (86.6 %±3.4) following incubation with 15.6μg/ml flunixin was observed with respect to the control (95.2 %±1.3). In the case of monocytes, a significant decrease (P≤0.05) in viability (59.1±7.8) was also observed in the second group of calves following exposure to flunixin at a concentration of 33μg/ml with respect to the control (73.3±8.2).

In the case of neutrophils, the lowest percentage of live cells was observed for cattle aged >1≤5 months following incubation with both concentrations of flunixin. In this group viability was 48.9 % and 48.7 %, and was statistically significant (P≤0.05) in comparison to the control (55.5 %).

The metabolic activity of the leukocytes incubated with flunixin was similar in all age groups irrespective of the concentration applied. The OD=550 values obtained ranged from 0.14 to 0.2 and the statistically significant (P≤0.05) effect in comparison to control was observed only in case of the leukocytes isolated from calves aged from 6 to 9 months (gr. 3). There was no interaction between the age of calves and dose of flunixin (Fig. 1).

In the present study, flunixin exhibited a slight inhibitory effect on the chemotaxis of the cells. Statistically significant (P≤0.05) inhibition of neutrophils migration in comparison to control was observed in the case of leukocytes isolated from all groups of cattle (Fig. 2). There was also a statistically significant inhibition of chemotaxis (P≤0.05) in comparison to control in case of treatment of the leukocytes with 15 μg/ml flunixin (Fig. 2).

Evaluation of the effect of flunixin on the production and release of nitrogen ions by the leukocytes revealed a decrease in NO concentration with respect to the control (4.5±2mmg/mM) and incubated with 15μg/ml and 33μg/ml flunixin. However, no significant effect of flunixin on the production and release of nitrogen ions by the leukocytes was observed (Fig. 3) Despite the differences in absolute values, the values obtained were not statistically significant and remained at a similar level, from 2.6 to 3.7 mg/mM.
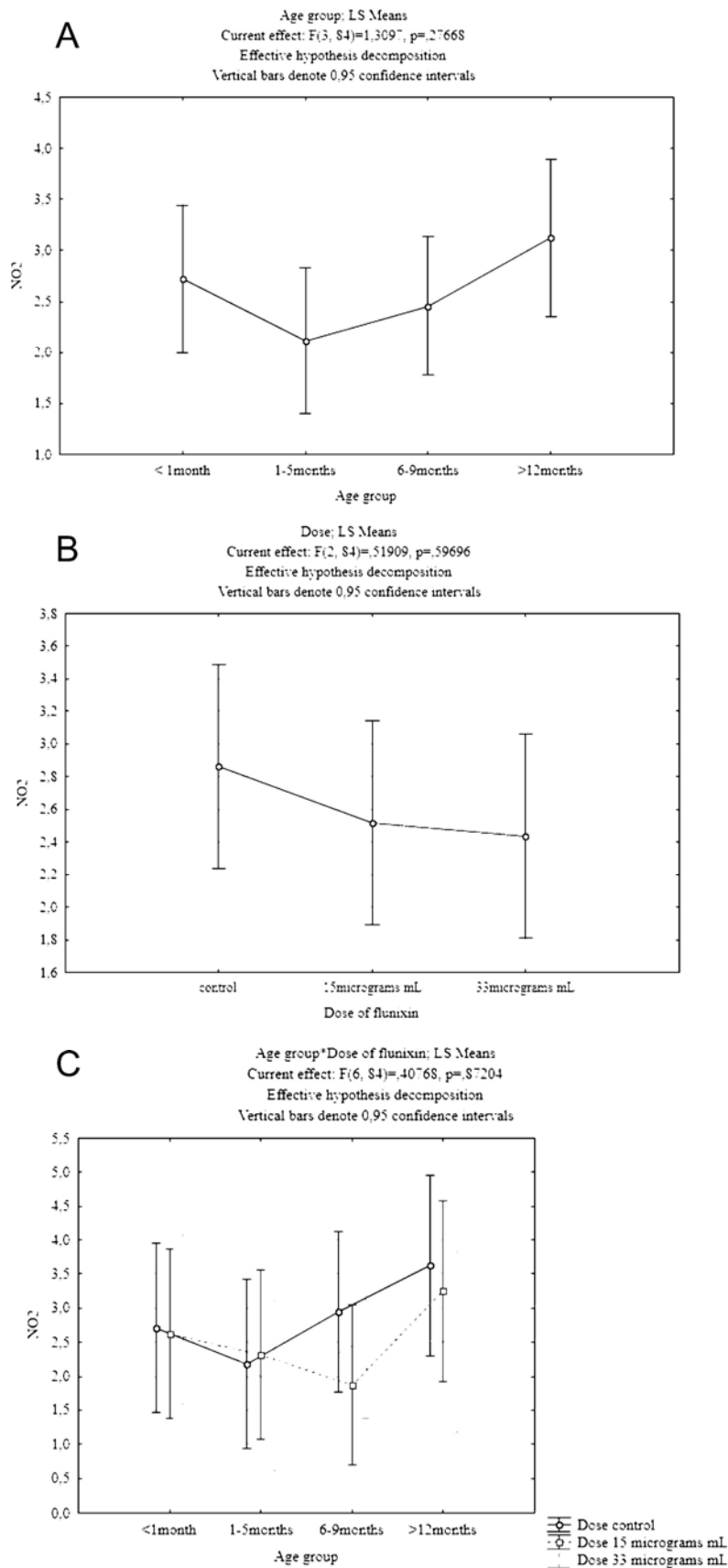
Fig. 1: **The statistical analysis of NBT assay results**

A- differences between the leukocytes isolated from calves on different age groups; B- differences between dose of flunixin; C-interactions between the groups and doses of flunixin.
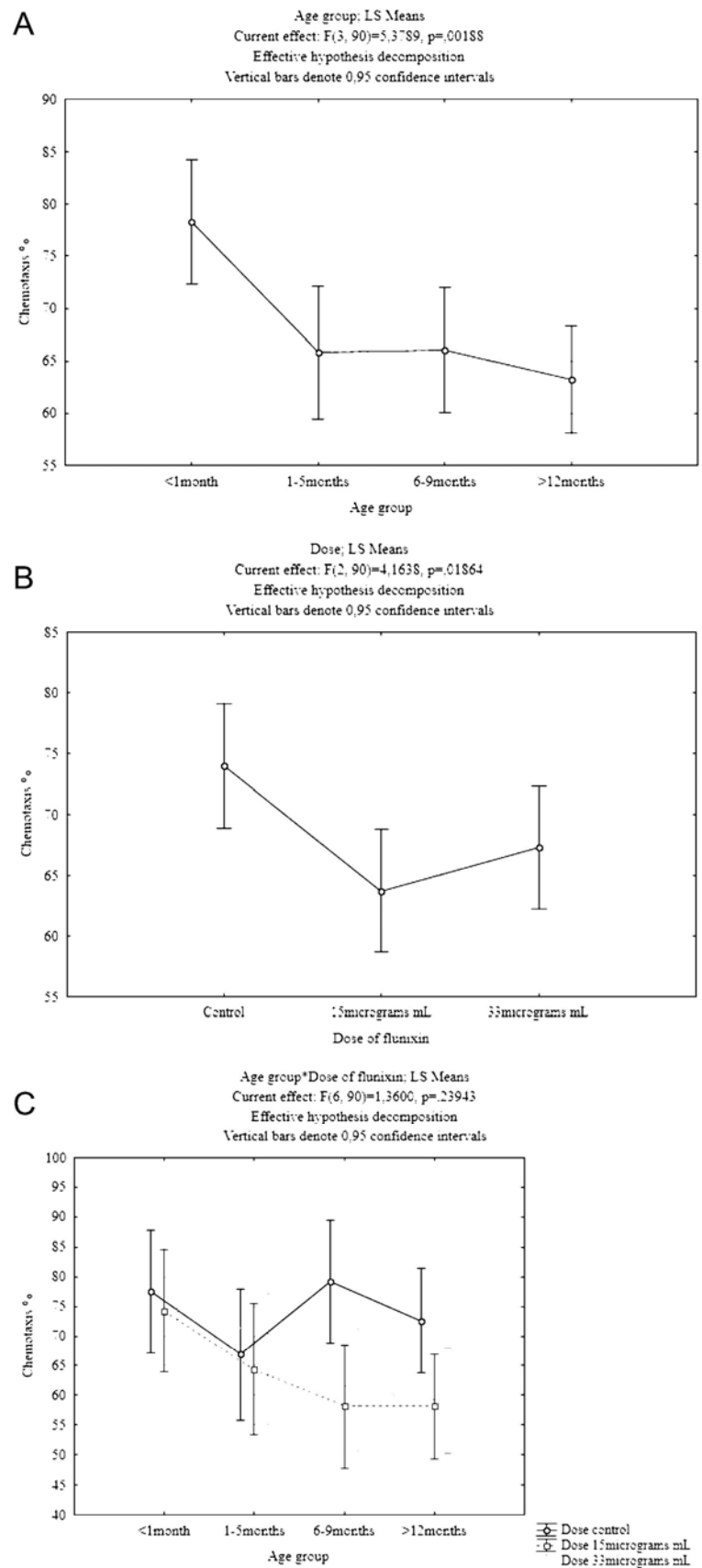
Fig. 2: **The statistical analysis of chemotactic activity results**
A- differences between the leukocytes isolated from calves on different age groups; B- differences between dose of flunixin; C-interactions between the groups and doses of flunixin.

Fig. 3: **The statistical analysis of NO$_2$ results assay**
A- differences between the leukocytes isolated from calves on different age groups; B- differences between dose of flunixin; C-interactions between the groups and doses of flunixin.

Fig. 4: **Apoptosis of leukocytes *in vitro* of different age groups depending on the concentration of flunixin**

A- differences between the leukocytes isolated from calves of different age groups; B- differences between dose of flunixin; C-interactions between the groups and doses of flunixin.
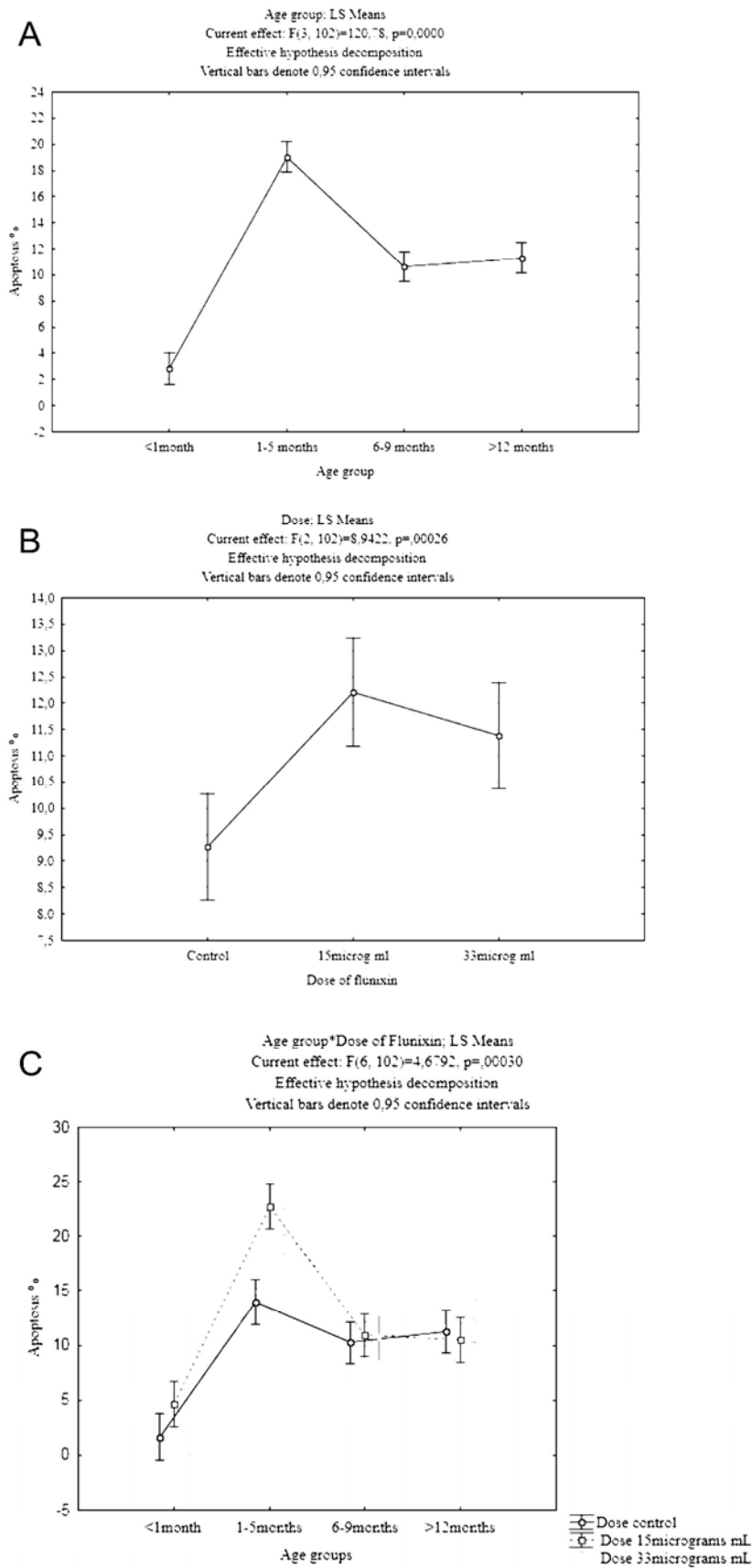
A significant correlation was observed in the present study between the concentration of flunixin and its effect on leukocytes. In the case of the NBT test, the correlation coefficient was higher than 0.87, while the correlation between nitrogen ion production and flunixin concentration was r≥0.67. The lowest correlation was observed between flunixin concentration and the chemotactic activity of the cells. The correlation coefficient was r=0.6 for the 33µg/ml concentration, while following incubation with 15.6µg/ml flunixin it was r=0.2 (Table 1).

Table 1. **Correlation coefficients between flunixin concentration and the estimated parameters**

Estimated parameters	Flunixin concentration	
	15.6µg/ml	33µg/ml
NBT		
Control	0.95	0.98
15.6 µg/ml	-	0.87
NO		
Control	0.7	0.67
15.6 µg/ml	-	0.7
Chemotaxis		
Control	0.2	0.6
15.6 µg/ml	-	0.02

Analysis of the influence of flunixin on apoptosis of the leukocytes showed the strongest effect of flunixin at concentrations of 15.6µg/ml and 33µg/ml in leukocytes isolated from the youngest calves (gr. 1) and calves from the second group. The values obtained were statistically significant (P≤0.05) with respect to the control (Fig. 4). For cells incubated with 15.6µg/ml flunixin, the average percentage of cells undergoing apoptosis was 4.7% in the case of the youngest calves and 22.3% in leukocytes isolated from group 2. In the case of leukocytes obtained from cattle aged from 6 to 9 months (gr. 3) and >1 year (gr. 4), the percentage of apoptotic cells did not change significantly in comparison to control. The sensitivity of cells depended mainly on the age of the animals. In the case of evaluation of the effect of a particular concentration of flunixin on induction of apoptosis, significantly significant differences were observed in the leukocytes isolated from the youngest calves (Fig. 4). The significant differences (P≤0.05) in leukocytes apoptosis has been also observed between the youngest calves and the remained groups of calves. However there was no significant differences in apoptosis between leukocytes obtained from calves aged from groups 3 and 4. The significant differences in leukocytes apoptosis has been also observed in case of dose of the flunixin.

The high correlation coefficient of r=0.7 between flunixin concentration and the percentage of cells undergoing apoptosis in the youngest group of calves indicates a significant relationship between the concentration of flunixin and its inductive effect on apoptosis in leukocytes. In the remaining age groups, the correlation coefficients ranged from 0.5 to 0.6, indicating a significant correlation between the amount of flunixin

and induction of apoptosis.

Discussion

The study demonstrated varied effects of flunixine meglumine on viability, apoptosis, oxidative stress, intracellular metabolism and chemotactic activity of leukocytes isolated from cattle of different ages. As showed these study experimental doses of flunixin did not significantly affect the total viability of the leukocytes, which remained at very high level in all age groups of calves. However in case of neutrophils and monocytes these studies showed a significant effect on decrease of viability in comparison to control in two groups of calves aged 1–5 and 6–9 months.

The slight decrease in total cell viability may indicate an insignificant effect of flunixin on mechanisms stabilizing intracellular structures in leukocytes. A study by Aydin and Üstüner (2009) found that doses exceeding the therapeutic concentration did not significantly contribute to chromosomal damage in leukocytes isolated from mice, which indicates that the toxic effect of flunixin on immune cells is slight.

However authors confirmed the influence of flunixin on the metabolism of the leukocytes expressed in the NBT, which was connected with the age of calves.

In the present study, flunixin exhibited an inhibitory effect on the chemotaxis of the cells. Statistically significant inhibition of neutrophils migration was observed in the case of leukocytes isolated from all groups of cattle. Moreover a significant inhibition of chemotaxis was observed in case of leukocytes treated with 15 µg/ml flunixin, which confirms a dependence of the impact mechanism of the drug dose. It is really interesting that these result was not observed after treatment of the leukocytes with 33 µg/ml flunixin.

A significant inhibitory effect of flunixin on chemotaxis was also noted for human leukocytes in a study by Kankaanranta et al. (1993). Another study (Maeda et al., 2011) found a strong suppressive effect of high concentrations of flunixin (50µg/ml) on proliferation of leukocytes obtained from calves and on expression of receptors for IFNγ, IL-2 and IL-4.

One unfavourable phenomenon observed in the effect of flunixin on leukocytes was its induction of apoptosis of cells from cattle of all age groups. However the strongest apoptotic effect of flunixin was observed in leukocytes obtained from the youngest calves and calves from the second group.

A similar study by Maślanka et al. (2010) conducted on leukocytes isolated from calves aged >1≤5 months found no significant effect on apoptosis in peripheral blood cells.

A significant difference of apoptosis has been also observed between the youngest calves and the remained groups of calves. The results obtained indicate that leukocytes isolated from the youngest calves and from calves aged >1 ≤ 5 months are most sensitive to flunixin. In older cattle neither of the flunixin concentrations applied significantly affected apoptosis in the cells. The varied effect of flunixin on apoptosis of leukocytes isolated from cattle of different ages is also indicated by

the statistically significant differences observed between groups, particularly in the group of youngest calves, cattle aged 6–9 months and over 12 months.

Conclusions

To sum up, it can be concluded that flunixin negatively affects the metabolic activity and viability of leukocytes, and its negative effect is associated with induction of apoptosis in leukocytes isolated from calves in all examined groups of calves.

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