Effects of flunixin and florfenicol combined with vitamins E and/or C on selected immune mechanisms in cattle under conditions of adaptive stress

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Received: November 11, 2014 Accepted: June 08, 2015

Abstract

The aim of the study was to evaluate the effect of flunixin and florfenicol administered in combination with vitamin E or C on selected leukocyte immune mechanisms and on the inflammatory process during the first few weeks in the feedlot. Fifty calves divided into 5 groups (n = 10) received florfenicol and flunixin with vitamin E or C. Blood was collected on the 1st, 3rd, 7th, 14th, 21st, and 28th d of the experiment. Intracellular metabolism (NBT), apoptosis, chemotaxis, susceptibility to M. haemolytica leukotoxin, and expression of β₂-integrins were determined in leukocytes. The symptoms of respiratory tract infection were observed in 40% of calves in control group, while in the other groups the morbidity rate ranged from 10% to 20%. Leukocytes showed decreased NBT, and the mean values for apoptosis ranged from 14% to 24%. The lowest percentage of apoptotic cells was observed in the calves that received florfenicol with flunixin and vitamins E and C. The chemotactic activity confirmed the significant inhibitory effect of the preparations on migration of the cells. A significant decrease (P ≤ 0.05) in the susceptibility of leukocytes to leukotoxin was noted in the group that received florfenicol and flunixin with vitamin E. Expression of β₂-integrin receptors was the lowest in calves receiving florfenicol with flunixin and vitamin E or C. The application of an antibiotic and a non-steroidal anti-inflammatory drug with antioxidants protected the leukocytes involved in defence against M. haemolytica virulence factors and effectively limited oxidative stress in the calves.

Keywords: calves, respiratory diseases, apoptosis, florfenicol, flunixin, vitamin E, vitamin C, cellular immunity.

Introduction

Bovine respiratory disease complex (BRDC) is a serious health and economic problem in feedlot cattle herds due to the involvement of numerous etiological agents, species-specific predispositions, and production technology.

Herd losses (16%-30%) resulting from increased morbidity rate, growth inhibition, reduced weight gain, decreased feed consumption and conversion (14%-20%), as well as high costs of prevention and treatment necessitate comprehensive measures to reduce economic losses (22, 23). In Great Britain, the total costs incurred for prevention and treatment of respiratory syndrome in cattle are about £60 million a year, while in the European Union annual expenditures equal €576 million (5).

Numerous infectious agents are involved in the aetiology of BRDC, including bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3), bovine viral diarrhoea virus (BVDV), herpesviruses BHV-1 and BHV-3, coronaviruses, and bacteria such as Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, Klebsiella pneumoniae, Actinobacillus spp., and mycoplasmas (3, 10, 11, 23, 30). A fundamental role in the aetiopathogenesis of the syndrome is played by viruses, which damage cells of the ciliary epithelium and impair phagocytosis...
mechanisms, preceding the development of pathological changes most often induced by bacteria. The immunosuppressive effect of viruses involves inhibition of neutrophil migration and activity, proliferation of B cells, impairment of NK cell activity, induction of apoptosis in CD4+ T cells, and induction of the production and release of proinflammatory cytokines (IL-1, 8, TNFα). Impairment of defence mechanisms of the lungs and the efficiency of bronchus-associated lymphoid tissue creates favourable conditions for the colonisation and development of bacteria, as well as for the expression of their pathogenic factors (12, 15).

Environmental and infectious agents usually play a synergistic role in the disease process. The most important predisposing factors are: stress associated with transport (excessive speed, noise, vibrations), microclimatic parameters, limited access to water, social stress, loading and unloading, and excessive physical exertion associated with maintaining a standing position (9, 19, 28).

An increased morbidity rate in calves with symptoms of BRDC occurs during the first 45 d in the feedlot. One of the factors determining morbidity is body weight upon entering the feedlot. Calves with low body weight (<180 kg) are more susceptible to illness, partly due to a decreased resistance to the negative effect of environmental stress factors associated with transport and adaptation (4, 19).

The aim of the study was to evaluate the effect of flunixin with florfenicol in combination with vitamin E and/or C on selected leukocyte defence mechanisms and on the course of the inflammatory process in cattle during the first few weeks in the feedlot.

Material and Methods

**Animals.** The study was performed on a group of 50 young Simmental beef cattle (males), aged 6 months, with a body weight of about 160 kg. Prior to entering the feedlot, all animals underwent an evaluation of their clinical condition. In addition, ELISA (Cypress Diagnostics, Belgium) was conducted to evaluate antibodies against bovine respiratory syncytial virus (BRSV), and the immunodiffusion test TRU RSV (Meridian Sciences, Belgium) was used for direct detection of the F and G genes of the virus in upper respiratory tract secretions. Both tests were performed and interpreted according to the manufacturer’s recommendations.

During the feedlot period, the animals received total mixed rations – TMR (ground grain, rapeseed, rolled cereals, hay, straw, spent grain, and maize silage) in amounts ranging from 3.5 to 4 kg, together with 2 kg CJ feed mix per calf, as well as hay ad libitum.

The animals were divided into 5 equal groups. Group 1 was the control. The animals in the experimental groups received once, on the first day in their feedlot, florfenicol and flunixin in combination with vitamin E or C (groups 2 and 3), florfenicol and flunixin in combination with vitamins E and C (group 4), or florfenicol and flunixin without vitamin supplements (group 5). The animals were clinically examined, including measurements of rectal temperature, on days 3, 7, 14, 21, and 28 in the feedlot.

Blood was collected for sera and into EDTA tubes (to obtain the cell fraction) on the day the preparations were administered and on days 3, 7, 14, 21, and 28 of the experiment.

**Isolation of leukocytes.** Leukocytes were isolated by density gradient separation using Histopaque-1083, according to an earlier study (26). The cell suspension obtained, with a density of 5 × 10^6 cells/mL, was suspended in RPMI 1640 medium with 7% FBS, penicillin G (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 10 mM solution of iodoacetamide (Sigma, Germany) containing 1 mg/mL of NBT dye (Sigma-Aldrich, Germany). Absorbance was read using a microplate reader (BioRad, model 680, USA) at a wavelength of 550 nm.

**Nitrotetrazolium blue reduction assay.** The metabolic activity of the leukocytes was determined by nitrotetrazolium blue reduction (NBT), according to an earlier study by Urban-Chmiel et al. (25). A cell suspension with a density of 5.5 × 10^6 cells/mL was cultivated for 1.5 h at 37°C in a 10 mM solution of iodoacetamide (Sigma, Germany) containing 1 mg/mL of NBT dye (Sigma-Aldrich, Germany). Absorbance was read using a microplate reader (BioRad, model 680, USA) at 488 nm (20).

**Leukocyte apoptosis.** Apoptotic cells were identified using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™), according to the procedure recommended by the manufacturer. Cytometric analysis was performed at 488 nm after adding 400 μL of 10% Annexin V Binding Buffer to each sample.

**Evaluation of the chemotactic activity.** 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 under an optical microscope (Olympus, Japan) at 40 x objective magnification. Chemotactic activity (%) was determined according to Alves et al. (2).

**Leukocyte susceptibility to M. haemolytica leukotoxin.** Leukocyte sensitivity to leukotoxin (Lkt) was determined by the MTT assay, according to Vega et al. (27). The Lkt unit was calculated as the reciprocal of the culture supernatant dilution causing a 10% death of bovine leukocytes. Each well of the microplate (Nunc) was filled with 100 μL of leukocyte suspension with a density of 2.5 × 10^6 cells/mL. The plate was incubated at 37°C for 1 h, and then 100 μL of Lkt in RPMI 1640 medium was added to each well. Following incubation (45 min at 37°C), 20 μL of MTT dye (5 mg/mL) (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, Sigma, Germany) was
added to each well and the plate was incubated at 37°C for 4 h. Then 100 µL of 0.04M HCl solution in isopropanol alcohol was added to each well. To test for non-specific reactions, samples were prepared using RPMI 1640 medium with MTT dye and cell suspension alone with MTT dye. The results were analysed using a Model 680 microplate reader (BioRad, USA) at 550 and 630 nm. Lkt was obtained from the supernatant of a reference strain of M. haemolytica serotype 1, according to Urban-Chmiel et al. (24).

**Expression of β₂-integrin receptors.** Expression of β₂-integrin receptors (CD11a/CD18) was evaluated using flow cytometry, according to Leite et al. (13). Cells suspended in PBS (pH 7.4) at a density of 2 × 10⁷/mL were incubated with bovine anti-CD18 antibodies (BAT 75A, VMRD, USA) diluted 1:100 in PBS with 5% BSA. The cells were washed in PBS with 1% bovine albumin (BSA, Sigma, Germany) and incubated in FITC-conjugated anti-IgG mouse monoclonal antibodies (BioKom, UK), diluted 1:100, for 30 min at room temperature, and then analysed in a cytometer (FAX, Beckman, USA). The results were expressed as the percentage of the cells with active receptors present in the cell membranes.

**Statistical analysis.** Statistical analysis was performed using Statistica 10.0 software (Statsoft, USA). The results were analysed by one-way ANOVA to compare differences between treatments, and the post-hoc differences were measured using Tukey’s test. Correlation of the results was analysed using Pearson’s coefficient. The correlations between parameters were determined using Pearson’s linear correlation coefficient. Differences were considered significant at P < 0.05.

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**Results**

Other than sporadic coughing, no significant clinical symptoms of respiratory syndrome were observed in the animals before they entered the feedlot. Average values for core body temperature were within physiological ranges for this species. Tests for the presence of BRSV in the herd showed positive results in ELISA and in the TRU RSV immunodiffusion test. The percentage of positive titres was 28% and 32% respectively, which confirms infection with this virus in the group of young beef cattle.

Among 50 animals studied, clinical respiratory symptoms (serous exudate from the nose, intense coughing, dyspnoea) and a body core temperature over 39.5°C were observed in 40% of calves from the control group between days 3 and 14 in the feedlot (group 1). The total morbidity rate in all animals was 16%, while mortality was 4% and affected only the control group.

NBT assay used to determine the intracellular metabolism of leukocytes collected on different days of the feedlot period showed a statistically significant (P ≤ 0.05) reduction in absorbance in groups 2 and 4 in comparison with the controls on days 3, 7, 14, and 21. In the case of group 4, the differences in mean absorbance values were statistically significant (P ≤ 0.05) in comparison with the other experimental groups (Fig. 1). A significant reduction in NBT in comparison with the control group was also observed in group 5 on days 7 and 21 and in group 3 on days 14 and 21 (Fig. 1). The highest absorbance values, ranging from 0.4 to 0.56, were observed on day 3.

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**Fig. 1.** Mean absorbance values obtained in the NBT test for leukocytes isolated from calves on different days in the feedlot (S ± SD)

* P ≤ 0.05 in comparison to control; Y axis – absorbance 550 nm; X axis – days in feedlot

** P ≤ 0.05 among experimental groups
The average values for cellular apoptosis in the experimental groups on different days of the feedlot period ranged from 14% to 24% (Table 1). The lowest percentage of apoptotic cells was observed in the animals from group 4. The highest percentage of apoptotic cells was observed in group 5 which received florfenicol and flunixin without vitamins. The results obtained were not statistically significant in comparison with the controls (Table 1). However, statistically significant differences (P ≤ 0.05) were observed between group 5, which received florfenicol with flunixin without of vitamins, and group 4, which received flunixin and florfenicol with vitamins E and C (Table 1.)

The chemotactic activity of neutrophils in different groups of calves confirmed that the preparations administered to the calves had a significant effect on cell migration induced by the chemotactic factor. A statistically significant (P ≤ 0.05) reduction in chemotaxis, in comparison with the control group, was observed on days 3 and 7 of the feedlot period in the calves which received florfenicol and flunixin, and on days 21 and 28 in those which received florfenicol and flunixin with vitamin C (Fig. 2). Statistically significant differences in the chemotaxis of cells, in comparison with the remaining experimental groups, were observed in group 5 on day 3 and in group 3 on days 21 and 28 (Fig. 2).

A significant reduction (P ≤ 0.05) in leukocyte susceptibility to Lkt, in comparison with the controls, was observed in group 2 on all days of the experiment (Fig. 3). For group 3, a significant reduction (P ≤ 0.05) in leukocyte susceptibility to Lkt was observed on days 7, 14, and 21 in the feedlot. In the case of group 5, significant differences were observed on days 14 and 21 (Fig. 3). In the remaining groups, despite differences in absolute values, there were no statistically significant differences in comparison with control group. In the case of group 2, statistically significant differences (P ≤ 0.05) in the susceptibility of leukocytes to Lkt were also noted on day 3 in comparison with the other experimental groups (Fig. 3).

Table 1. Average apoptosis of leukocytes obtained from calves on particular days in the feedlot (mean ± SE)

<table>
<thead>
<tr>
<th>Days in feedlot</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>19.3 ± 4.5</td>
<td>17.2 ± 3.1</td>
<td>18.5 ± 4.5</td>
<td>16.4 ± 5</td>
<td>16.2 ± 5.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>16.6 ± 4.8</td>
<td>19.7 ± 5.4</td>
<td>18.4 ± 4.4</td>
<td>17.4 ± 4.8</td>
<td>15.8 ± 5.2</td>
</tr>
<tr>
<td>Group 3</td>
<td>17.1 ± 3.8</td>
<td>16.8 ± 4.1</td>
<td>16.5 ± 3.8</td>
<td>15.8 ± 4.2</td>
<td>15.9 ± 4.5</td>
</tr>
<tr>
<td>Group 4</td>
<td>15.4 ± 4.2</td>
<td>15.5 ± 5.5</td>
<td>14.1 ± 4.8</td>
<td>14.4 ± 6.2</td>
<td>14.3 ± 1.2</td>
</tr>
<tr>
<td>Group 5</td>
<td>23.9 ± 5.6**</td>
<td>21.2 ± 2.9**</td>
<td>19.8 ± 4.1**</td>
<td>19 ± 4.8**</td>
<td>17.8 ± 4.2**</td>
</tr>
</tbody>
</table>

** P ≤ 0.05 among the experimental groups

Fig. 2. Chemotaxis of the cells isolated from calves on different days of the feedlot period (S±SD)

* P ≤ 0.05 in comparison with the control group

** P ≤ 0.05 in comparison with other experimental groups
Fig. 3. Susceptibility of leukocytes to *M. haemolytica* Lkt on different days in the feedlot
* P ≤ 0.05 in comparison with the control group

Fig. 4. Expression of β₂-integrin receptor fragments (CD18/CD11a) in the membranes of leukocytes isolated from calves on different days in the feedlot
* P ≤ 0.05 in comparison with control group
** P ≤ 0.05 in comparison with group 4

Table 2. Correlation coefficients between expression of β₂-integrin receptors and leukocyte susceptibility to *M. haemolytica* Lkt (r < 0.05)
F-florfenicol, Fl- flunixin

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (Gr. 1)</th>
<th>F+Fl+vit.E (Gr. 2)</th>
<th>F+Fl+vit.C (Gr. 3)</th>
<th>F+Fl+vit.E&amp;C (Gr. 4)</th>
<th>F+Fl (Gr. 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>0.72*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.74*</td>
<td>0.8*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.72*</td>
<td>0.6*</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.74*</td>
<td>0.6*</td>
<td>0.4</td>
<td>0.7*</td>
<td>-</td>
</tr>
</tbody>
</table>

* Significant correlation

Furthermore, immunofluorescent staining and flow cytometric analysis revealed the differences in the expression of β₂-integrin receptors in leukocytes of calves from different experimental groups. The percentage of expression of β₂-integrin receptors was the lowest in the animals which received florfenicol and flunixin in combination with vitamin E or C (groups 2 and 3); the values obtained were ≥50%
(Fig. 4). Statistically significant differences in comparison with the controls were observed in group 2 on days 3, 7, and 14, and in group 3 on day 14-in the feedlot. A significant \( P \leq 0.05 \) decrease in the expression of \( \beta_2 \)-integrin receptors in leukocyte membranes was also observed in the group receiving florfenicol and flunixin without antioxidants (group 5) on days 7 and 14. Moreover, in comparison with group 4, statistically significant differences were noted in the expression of \( \beta_2 \)-integrin receptors in group 2 on days 3, 14, 21, and 28 in the feedlot, and in group 3 on days 3 and 28.

The analysis of the correlation coefficients between the expression of \( \beta_2 \)-integrin receptors and leukocyte susceptibility to Lkt showed that these parameters were correlated in all experimental groups. The coefficients had values of \( r > 0.7 \). The correlations in different experimental groups ranged from \( r = 0.4 \) to \( r = 0.8 \) (Table 2).

**Discussion**

The study showed a varied inhibitory effect of florfenicol and flunixin, alone or in combination with vitamins E and/or C, on cellular mechanisms of systemic defence, which resulted in reduced chemotaxis of neutrophils on different days in the feedlot. The strongest protective effect of leukocytes against the cytotoxic effect of *M. haemolytica* Lkt was observed following the application of florfenicol and flunixin in combination with vitamin E. This resulted in a significant decrease in the susceptibility of cells to Lkt, correlated with a decrease in the expression of *M. haemolytica* receptors of \( \beta_2 \)-integrin fragments CD18/CD11a, which are specific for leukotoxin (27). It should be noted that increased susceptibility of leukocytes to leukotoxin is a key element in the development of respiratory syndrome induced by *M. haemolytica* in cattle.

The changes observed may be due to the inhibition of the inflammatory reaction, which contributed to the reduction in the morbidity rate during the first few weeks in the feedlot. Infiltration of neutrophils induced by proinflammatory factors in the early stage of inflammation has been shown to be a significant element of the defence process. A prolonged process of leukocyte activity increases the amount of proteolytic enzymes and free radicals released, and the latter in turn increase damage to cells involved in protection against respiratory infections (1, 18). In a study conducted on cows in the perinatal period, Pinotti *et al.* (14) observed a slight increase in chemotactic activity following administration of \( \alpha \)-tocopherol, which, according to the authors, may be the effect of systemic immunomobilisation in the cows during this period. Another study (16) confirmed that antioxidants may inhibit the inflammatory response within pulmonary tissue, resulting in reduced chemotaxis and phagocytosis of neutrophils isolated from mice.

The positive effect of the preparations used is evidenced by the results obtained in the evaluation of the intracellular metabolism of leukocytes with the NBT test. The statistically significant \( P \leq 0.05 \) reduction in absorbance on different days of the feedlot period indicates a beneficial effect on the stability of intracellular structures, which indirectly indicates a reduction in oxidative stress in the cells, expressed as decreased free radical production. According to Esfandiari *et al.* (8) and Sabeur and Ball (17), the reduction reaction of nitrotetrazolium blue can be used to evaluate the level of oxidative stress on the basis of the amount of oxygen radicals produced and released by the cells, which has been confirmed in studies on humans and rats.

The substantial increase in the resistance of leukocytes to the cytotoxic effect of *M. haemolytica* Lkt, accompanied by reduced expression of \( \beta_2 \)-integrin membrane receptors, confirms the significant effect of the preparations on the cells’ defence mechanisms directed against virulence factors of the bacteria. The high correlation \( r > 0.5 \) between the susceptibility of leukocytes to Lkt and expression of \( \beta_2 \)-integrin receptors may be a measurable indicator in determining the susceptibility of calves to infections induced by *M. haemolytica*. The protective effect on cells involved in the immune response in the lungs may significantly reduce morbidity in cattle with symptoms of respiratory syndrome.

The positive effect of the preparations is also evidenced by the approximately threefold lower morbidity rate in the young beef cattle in comparison with the controls. The results are similar to those obtained by Chirase *et al.* (6), who administered vitamins E and C to calves and achieved a substantial decrease in their morbidity and mortality rates in comparison to calves that did not receive antioxidants. Ekstrand-Hammarström *et al.* (7) demonstrated that administration of vitamin E can inhibit the inflammatory process by inhibiting the production of proinflammatory cytokines, reducing production of acute phase proteins and weakening the inflammatory response of epithelial cells in the lungs. Moreover, in a study by Weingarten *et al.* (29), in calves with symptoms of respiratory syndrome which were administered florfenicol and flunixin, a reduction in the disease process was observed in the bronchi and lungs, expressed as abatement of clinical symptoms.

To sum up, it should be emphasised that simultaneous administration of an antibiotic and a non-steroid anti-inflammatory drug together with antioxidants protected leukocytes involved in defence against the virulence factors of *M. haemolytica*. The preparations also effectively limited the development of oxidative stress and inflammatory process in the feedlot calves, resulting in increased resistance to disease. The
presented findings may be useful in development of effective adjuvant therapy.

**Conflict of Interests Statement:** The authors declare that there is no conflict of interest regarding the publication of this article.

**Animal Rights Statement:** The experiment was conducted in accordance with the Local Ethics Commission.

**References**