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Original article

The applicability of the Migratest® kit for evaluating the chemotactic activity of peripheral blood neutrophils in goats on the example of animals supplemented with β-hydroxy-β-methylbutyrate (HMB)

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Abstract

The objective of this study was to determine the applicability of the Migratest® kit for evaluating the chemotactic activity of peripheral blood neutrophils in goats. The experiment was performed on 14 goat kids aged 30±2 days, divided into two groups of 7 animals each: C - control group, and E – experimental group, supplemented with β-hydroxy-β-methylbutyrate (HMB), a typical immunostimulant which influences the phagocytic activity of peripheral neutrophils. The feed administered to experimental goat kids was supplemented with HMB at 40 mg/kg BW, whereas control goat kids were administered standard farm-made feed without supplementation. Blood was sampled from the jugular vein immediately before the experiment (day 0) and on experimental days 15, 30 and 60 to determine the chemotactic activity of peripheral blood neutrophils in goats. The results of the study indicate that the Migratest® kit can be used to evaluate the influence of immunomodulators on the chemotactic activity of peripheral blood neutrophils in goats. The results of the assay are most effectively presented by calculating the chemotactic index which accounts for the chemotaxis or migration of neutrophils in the presence or absence of a chemotactic factor, respectively, and the percentage of granulocytes that migrate towards fMLP. The results of both presentation methods appear to be identical.

Key words: goats, Migratest® kit, chemotactic activity of peripheral blood neutrophils, β-hydroxy-β-methylbutyrate

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Introduction

Phagocytosis is the most important non-specific immune response which constitutes the first line of defence in the body. There are two types of circulating phagocytic cells: neutrophils (polymorphonuclear leukocytes, PMN) and monocytes (mononuclear leukocytes, MN). Macrophages are non-circulating phagocytic cells which reside in tissues. Blood monocytes and tissue macrophages represent two different life stages of the same cell line (Abbas et al. 2009).

Phagocytosis is a multi-stage process which is composed of 4 major steps: chemotaxis and migration, adherence, engulfment, intracellular digestion and killing. As a result, the engulfed object is damaged and immune mechanisms are activated (Abbas et al. 2009).

Recent decades have witnessed considerable progress in analytical techniques, and flow cytometry emerged as a reliable, repeatable and highly sensitive diagnostic tool that is widely used in both human and veterinary medicine. Traditional spectrophotometric and microscopic methods are being gradually replaced by flow cytometry (Silveira 2015).

Flow cytometry is also applied to evaluate different stages of phagocytosis. The most popular commercial kits include the Phagoburst® kit for analysing the production of reactive oxygen species by phagocytes, the Phagotest® kit for evaluating phagocytes' ability to ingest bacteria, and the Migratest® kit for analysing phagocytes' ability to migrate towards a chemotactic stimulus. These kits have been designed for use in human medicine, but Phagoburst® and Phagotest® have also been used successfully in rats (Wójcik and Dąbkowska 2010), lambs (Wójcik 2014a) and calves (Wójcik 2014b). In the literature, the Migratest® kit was used only in one study of piglets (Krause et al. 2005).

The aim of this study was to determine the applicability of the Migratest® kit for evaluating the chemotactic activity of peripheral blood neutrophils in goats.

Materials and Methods

Experimental design

The experiment was performed on 14 goat kids aged 30±2 days, divided into two groups of 7 animals each: C - control group and E – experimental group. The feed administered to the experimental goats was supplemented with β-hydroxy-β-methylbutyrate (HMB, Metabolic Technologies Inc. Ames, IA, USA) at 40 mg/kg BW, whereas control goats were administered standard farm-made feed without supplementation. The study was conducted on non-supplemented control animals and experimental animals whose diets were supplemented with β-hydroxy-β-methylbutyrate (HMB), an immunostimulator with proven stimulatory effects on phagocytic activity in ruminants (Wójcik et al. 2013).

Blood was sampled from the jugular vein before HMB supplementation and on days 15, 30 and 60 of the experiment to determine and compare the chemotactic activity of peripheral blood neutrophils.

Evaluating the chemotactic activity of peripheral blood neutrophils

MIGRATEST® kit (Glycotope Biotechnology GmbH, Heidelberg, Germany) was performed according to the manufacturer's specifications. Leucocyte-rich plasma (LRP) isolated from heparinized whole blood by spontaneous sedimentation was used in the experiment. LRP from each sample was placed in two cell culture inserts with pore size of 3.0 µm. One insert was transferred to a well containing the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP). The other insert was placed in a buffer solution without chemotactic peptide as negative control. Chemotaxis was conducted for 30 min at 37°C. The cells were stained for 10 min with an antibody reagent (FITC-labelled anti-CD62L) containing counting beads. Before flow cytometry, a special vital DNA dye was added for 5 min on ice. The samples were analysed by flow cytometry to determine the number of migrated neutrophils and to measure L-selectin shedding of activated cells.

FACS acquisition and analysis

Flow cytometry was performed using a FACSCanto II cytometer (BD Biosciences, USA). Data were acquired with FACSDiva version 6.1.3 software (BD Biosciences, USA) and analysed by FlowJo 10 software (Tree Star, USA). The cytometry setup and tracking beads (CST; BD Biosciences, USA) were used to initialize the photomultiplier tube (PMT). Unstained control cells and single stain control for every fluorochrome were prepared and used to establish flow cytometric compensation.

Granulocytes and counting beads were identified in PerCP to SSC scatter and depicted in FSC to SSC scatter (Fig. 1). Data acquisition ended after the acquisition of exactly 2000 events in the region of counting beads. The number of events in the region of granulocytes was then counted, and the number of granulocytes in the control sample was compared with the number of granulocytes in the positive control sample after stimulation with fMLP. The decrease in L-selectin expression could be measured simultaneously. Downregulation

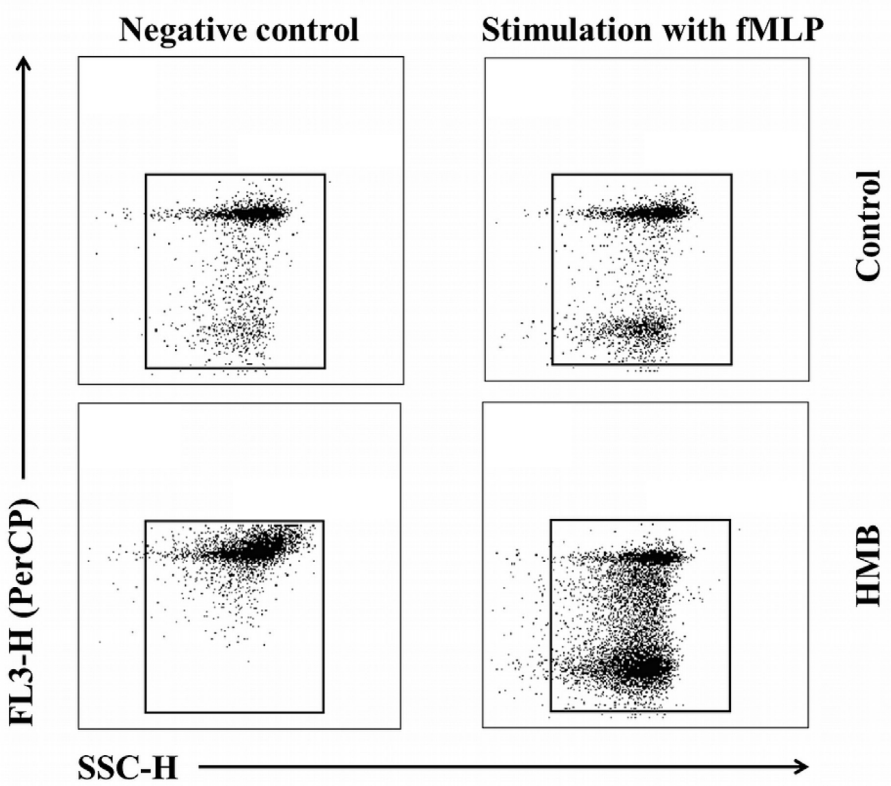

Fig. 1. Gating strategy for analysing flow cytometry data based on the neutrophil migration assay. Granulocytes and counting beads were identified in PerCP to SSC scatter and depicted in FSC to SSC scatter. **A** – control without stimulation; **B** – control stimulated with fMLP; **C** – HMB without stimulation; **D** – HMB stimulated with fMLP;

of this cell adhesion molecule correlated directly with the activation of neutrophils under exposure to chemotactic factors. Changes in cell shape preceded cell migration and could be measured by analysing changes in forward scatter signals during flow cytometry.

Statistical analysis

Numerical results were presented as the arithmetic mean ± SD. The obtained results were processed statistically by two-way ANOVA analysis of variance for orthogonal designs. In post-hoc analysis Dunnett's test was used to compare day 0 with day 15, 30 and 60 in group E (the significance of differences between days: A - $p<0.05$; B - $p<0.01$; C - $p<0.001$; D - $p<0.0001$), and Tukey's test for equal groups to compare group E with group C in each time point (the significance of differences between groups: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$) with the use of GraphPad Prism 7 software. A significance level has been set to 5%.

Ethics committee

Animal experiments were carried out in conformance with the Animal Protection Law (Journal of Laws of 24 February 2005, no. 33, item 289) and the recommendations of the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn. During the experiment, animals were kept in Faculty premises under adequate experimental conditions.

Results

The results of the Migratest® assay revealed a significant increase in the chemotactic activity of peripheral blood neutrophils in the experimental goats (E) supplemented with HMB relative to non-supplemented control goats (C) and relative to the average initial values (day), regardless of the method of data presentation (Figs. 2-9, 10, 11).

Discussion

The results of the Migratest® assay were presented in several variants based on an analysis of the available methods in the literature. These variants were divided into two main groups: direct methods and indirect methods.

In the group of direct methods, the results can be presented as the number of granulocytes that migrated towards fMLP (Schildhauer et al. 2009) or the number of granulocytes that migrated towards fMLP and the

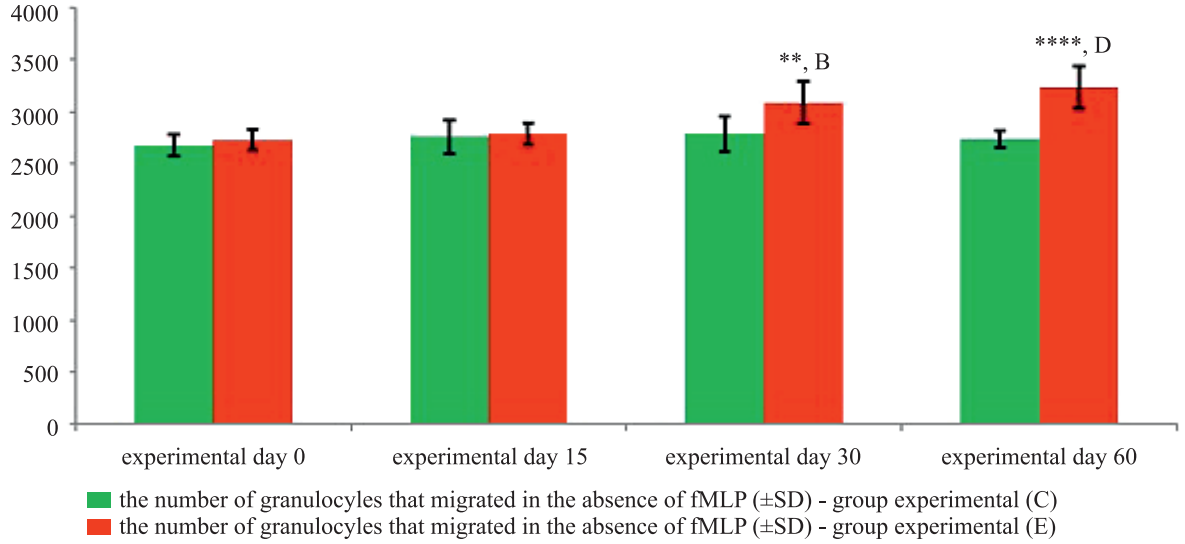

Fig. 2. Number of spontaneously migrating peripheral blood neutrophils in goats supplemented with HMB and non-supplemented control goats. When counting beads were gated in FSC/SSC scatter and 2000 counting beads were acquired in this region, sample flow was stopped, neutrophils were gated and counted in this region. The result corresponds to the number of neutrophils that migrated through cell culture inserts with a pore size of 3.0 µm in the absence of fMLP chemoattractant. On days 30 and 60, a significant increase ($p<0.05$ and $p<0.001$, respectively) in the number of spontaneously migrating neutrophils was observed in goats supplemented with HMB relative to the number of spontaneously migrating neutrophils in control group goats and relative to the average initial values (day 0).

Key: C – control group; E – experimental group; SD - standard deviation; Numerical results were presented as the arithmetic mean ± SD. A significance level has been set to 5%.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$;

A - $p<0.05$; B - $p<0.01$; C - $p<0.001$; D - $p<0.0001$ relative to day 0.

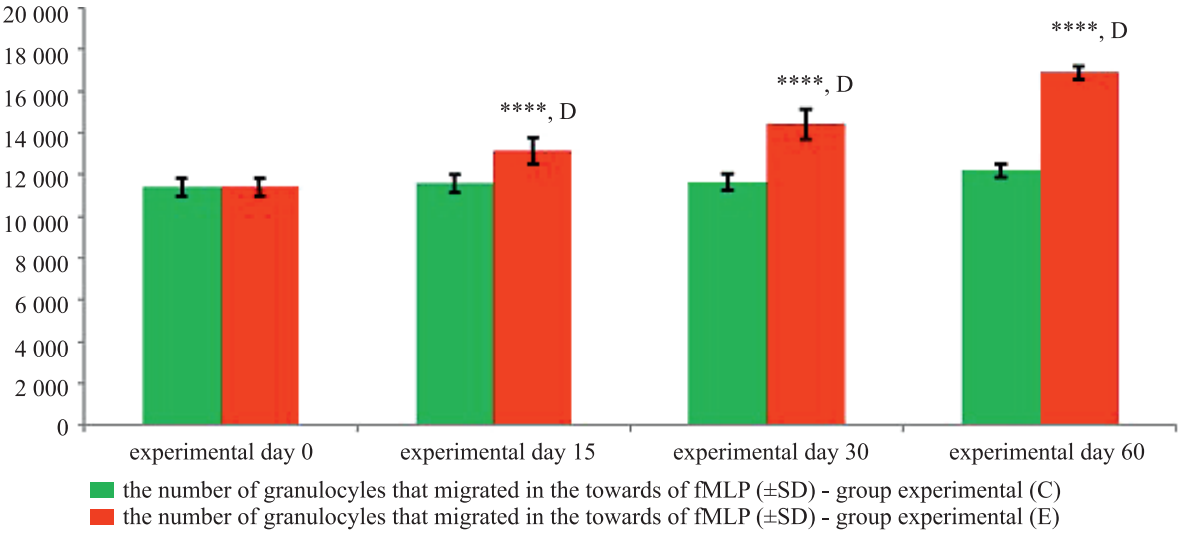

Fig. 3. Number of spontaneously migrating peripheral blood neutrophils in goats supplemented with HMB and non-supplemented control goats. When counting beads were gated in FSC/SSC scatter and 2000 counting beads were acquired in this region, sample flow was stopped, neutrophils were gated and counted in this region. The result corresponds to the number of neutrophils that migrated through cell culture inserts with a pore size of 3.0 µm towards a concentration gradient of fMLP chemoattractant. A significant increase ($p<0.001$) in the number of neutrophils migrating towards fMLP was observed throughout the entire experiment in goats supplemented with HMB relative to the number of neutrophils migrating towards fMLP in control group goats and relative to the average initial values (day 0).

Key: refer to Fig. 2.

number of granulocytes that migrated in the absence of fMLP (Álvarez-Rodríguez et al. 2013). When the results of this study were presented with the use of direct methods, the number of neutrophils that migrated towards fMLP increased significantly in goats supplemented with HMB relative to the control group throughout the entire experiment. A significant increase in the number of spontaneously migrating neutrophils

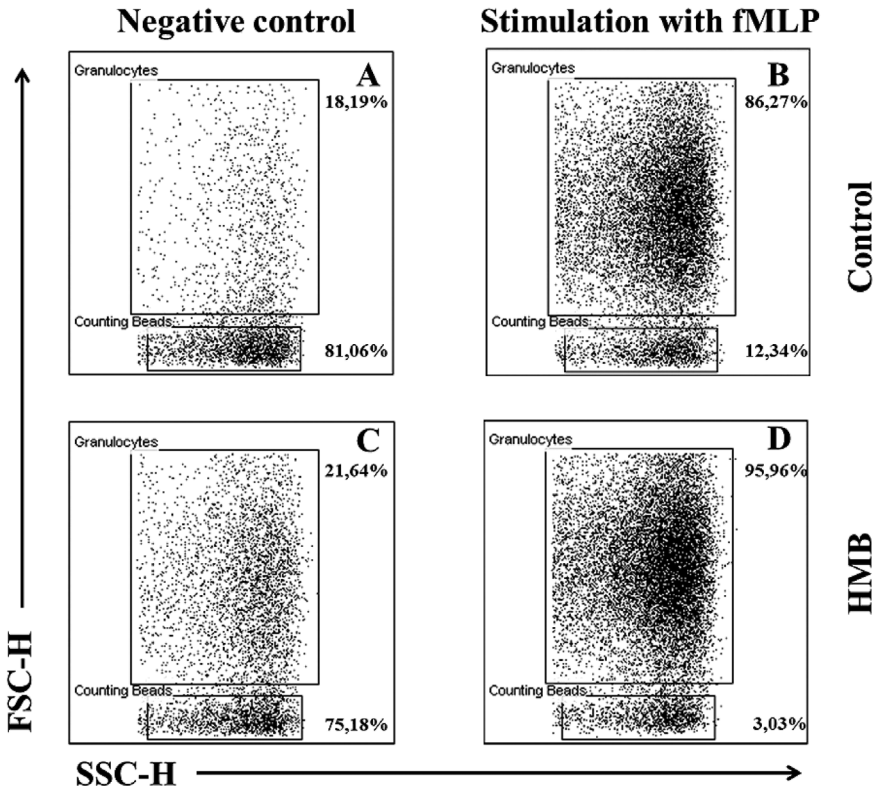

Fig. 4. Representative dot plot cytograms showing the percentage of migrating granulocytes and counting beads. A – control without stimulation; B – control stimulated with fMLP; C – HMB without stimulation; D – HMB stimulated with fMLP; The percentage of granulocytes relative to the number of whole cells was determined in each sample as soon as 2 000 counting beads were acquired. Stimulation with fMLP increased the number of migrated granulocytes (**B**, **D**) relative to the controls without fMLP (**A**, **C**). The chemotactic activity of spontaneously migrating granulocytes and granulocytes migrating towards fMLP increased in the experimental group (supplemented with HMB) relative to the non-supplemented control group.

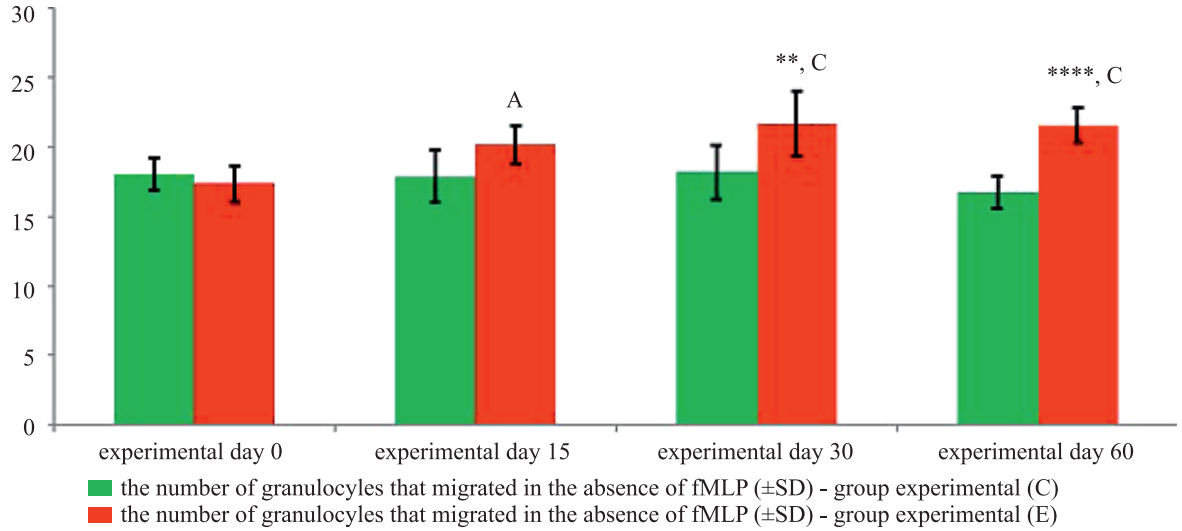

Fig. 5. Percentage of spontaneously migrating peripheral blood neutrophils in goats supplemented with HMB and non-supplemented control goats. When counting beads were gated in FSC/SSC scatter and 2000 counting beads were acquired in this region, sample flow was stopped, neutrophils were gated and their percentage was determined in the region. The result corresponds to the chemotactic activity of neutrophils, i.e. the number of neutrophils that migrated through cell culture inserts with a pore size of 3.0 µm in the absence of fMLP chemoattractant. On days 30 and 60, a significant increase ($p<0.05$ and $p<0.001$, respectively) in the number of spontaneously migrating neutrophils was observed in goats supplemented with HMB relative to the number of spontaneously migrating neutrophils in control group goats and relative to the average initial values (day 0).

Key: refer to Fig. 2.

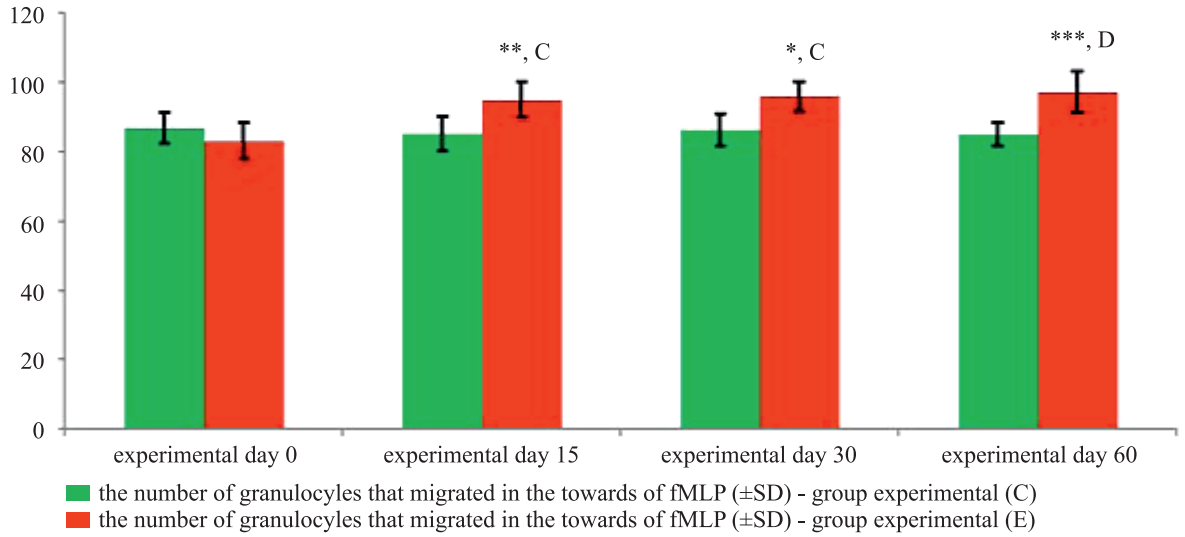

Fig. 6. Percentage of spontaneously migrating peripheral blood neutrophils in goats supplemented with HMB and non-supplemented control goats. When counting beads were gated in FSC/SSC scatter and 2000 counting beads were acquired in this region, sample flow was stopped, neutrophils were gated and their percentage was determined in the region. This percentage corresponds to the chemotactic activity of neutrophils, i.e. the number of neutrophils that migrated through cell culture inserts with a pore size of 3.0 µm towards a concentration gradient of fMLP chemoattractant. A significant increase ($p<0.01$ on days 15 and 30; $p<0.001$ on day 60) in the percentage of neutrophils migrating towards fMLP was observed throughout the entire experiment in goats supplemented with HMB relative to the percentage of neutrophils migrating towards fMLP in control group goats and relative to the average initial values (day 0).

Key: refer to Fig. 2.

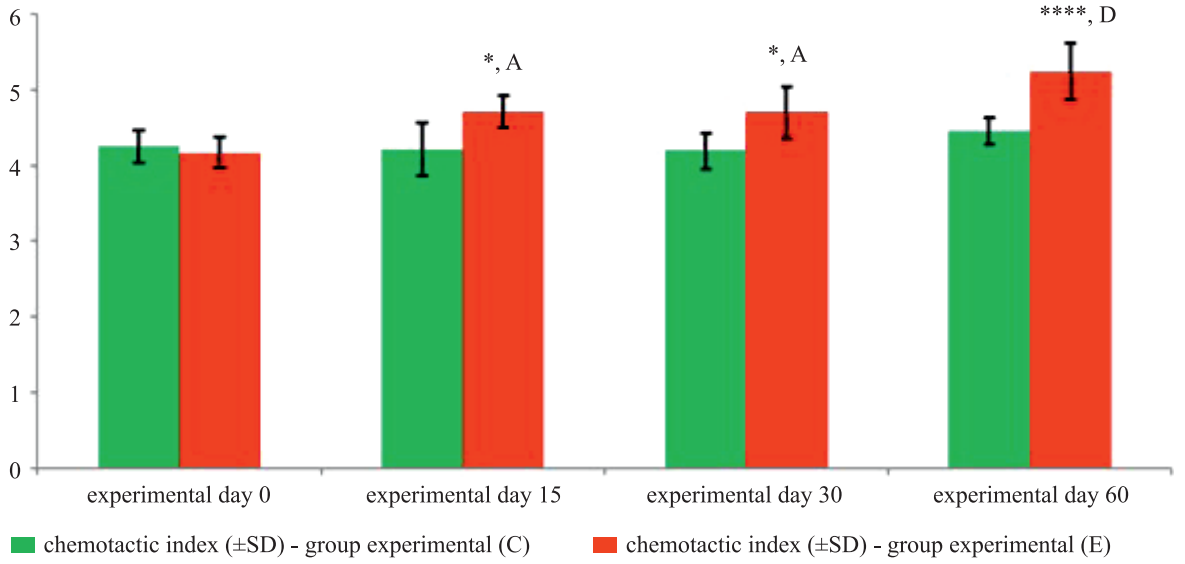

Fig. 7. The chemotactic index was calculated by dividing the number of cells that migrated towards fMLP by the number of cells that migrated in the absence of fMLP. The chemotactic index of neutrophils in goats supplemented with HMB was significantly higher ($p<0.01$ on days 15 and 30; $p<0.001$ on day 60) throughout the entire experiment relative to the chemotactic index of neutrophils in the non-supplemented control group and relative to the average initial values (day 0).

Key: refer to Fig. 2.

was observed only up to day 60 in the experimental group relative to the control group.

A direct method was also proposed by Hagelauer et al. (2015). In this approach, the results of the Migratest® assay are presented as the percentage of spontaneously migrating neutrophils and the percentage of neutrophils that migrate towards fMLP. This method was used to present our results in dot plot cytograms and diagrams. In the group of goats supplemented with HMB, the percentage of spontaneously migrating neutrophils was significantly higher on days 30 and 60 and the percentage of neutrophils migrating towards fMLP was significantly higher on days 15, 30 and 60 than in the non-supplemented control group.

Indirect methods involved the chemotactic index and Δ migrating cells. The chemotactic index was cal-

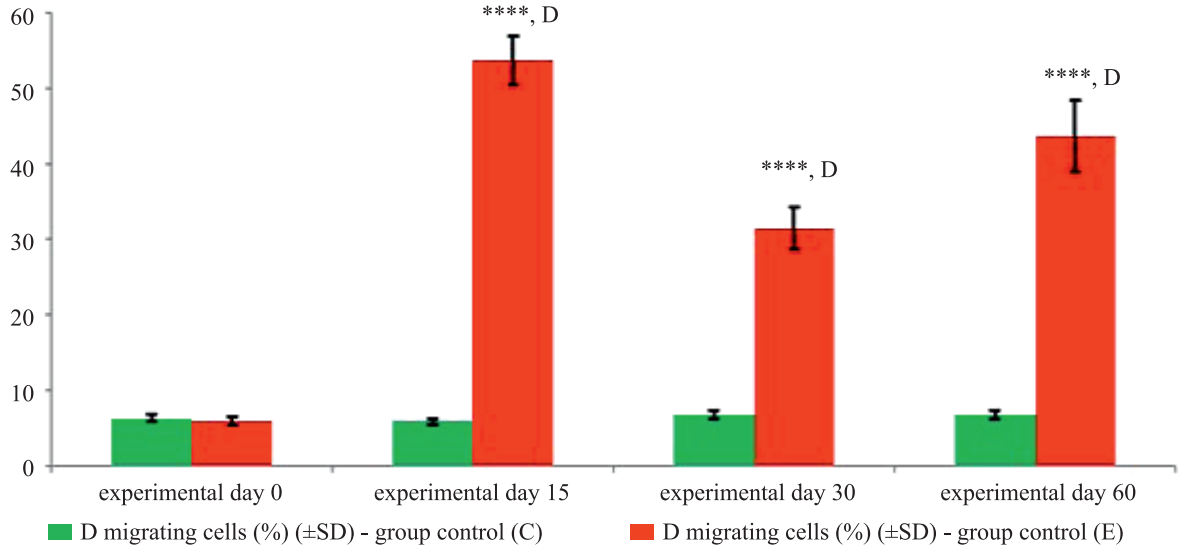

Fig. 8. Δ migrating cells calculated as the difference between the ratio of the percentage of migrating neutrophils and the percentage of counting beads in an fMLP-stimulated sample and the ratio of the percentage of migrating granulocytes and the percentage of counting beads in a non-stimulated sample. Throughout the entire experiment, a significant increase ($p<0.001$) in Δ migrating cells (%) was observed in the group of goats supplemented with HMB relative to the non-supplemented control group and relative to the average initial values (day 0).

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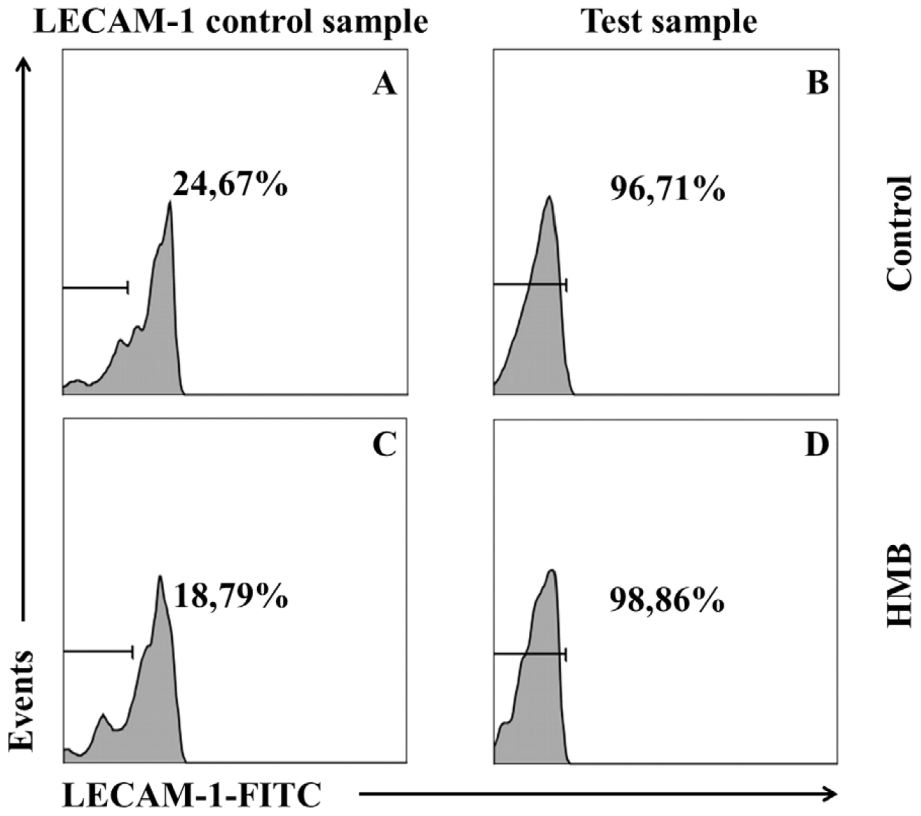

Fig. 9. Representative histograms showing L-selectin expression. **A** – control, LECAM-1 control sample; **B** – control, test sample; **C** – HMB, LECAM-1 control sample; **D** – HMB, test sample. The percentage of chemotactically activated neutrophils with decreased expression of L-selectin (LECAM-1) was determined. L-selectin expression decreased in LECAM-1 control samples (**A**, **C**) relative to test samples (**B**, **D**), and the decrease was correlated with an increase in the chemotactic activity of neutrophils. The decrease was more pronounced in the group of goats supplemented with HMB (**D→C**) than in the non-supplemented control group (**B→A**).

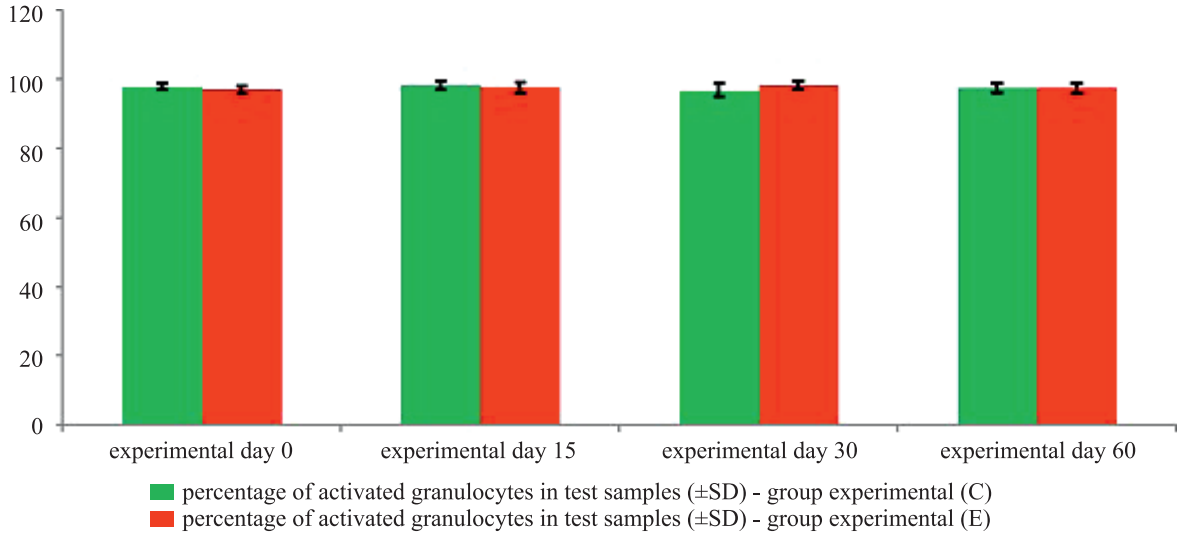

Fig. 10. Percentage of chemotactically activated neutrophils with decreased expression of L-selectin (LECAM-1) in test samples. FITC-labelled antibodies against the L-selectin antigen (CD62L), present on the surface of neutrophils, were added to samples, and the percentage of cells not expressing this antigen was determined. Fluorescence was not detected, therefore, these cells were chemotactically activated. In test samples, significant differences in chemotactic activity were not observed between neutrophils in goats supplemented with HMB and the non-supplemented control group.

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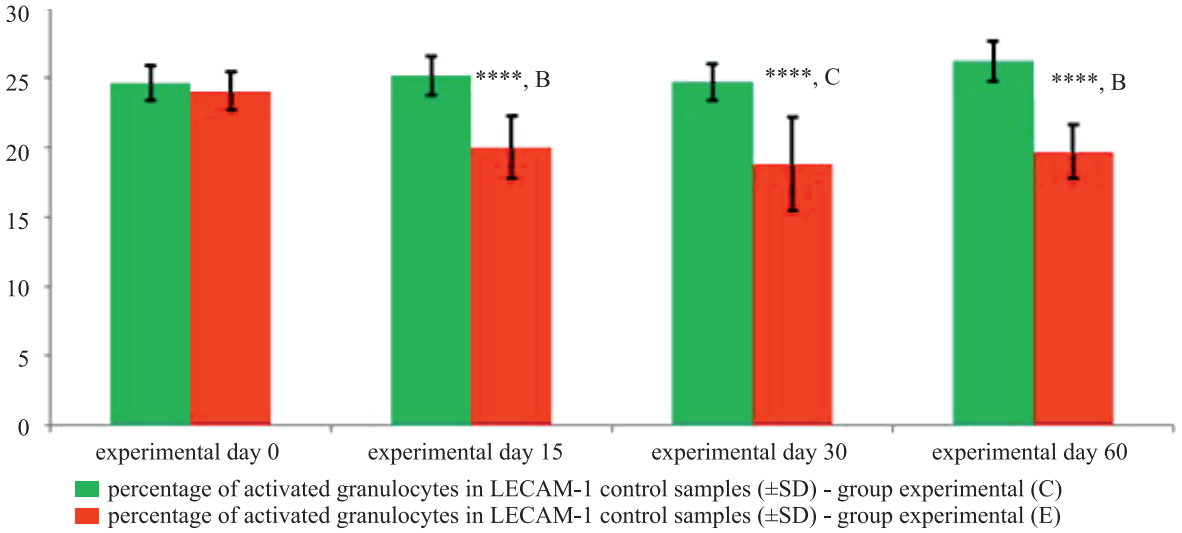

Fig. 11. Percentage of chemotactically activated neutrophils with decreased expression of L-selectin (LECAM-1) in LECAM-1 control samples. FITC-labelled antibodies against the L-selectin antigen (CD62L), present on the surface of neutrophils, were added to samples, and the percentage of cells not expressing this antigen was determined. Fluorescence was not detected, therefore, these cells were chemotactically activated. In LECAM-1 control samples, a significant increase in the chemotactic activity of neutrophils was observed throughout the entire experiment ($p<0.05$ on day 15; $p<0.01$ on days 30 and 60) in goats supplemented with HMB relative to the non-supplemented control group.

Key: refer to Fig. 2.

culated by dividing the number of cells that migrated towards fMLP by the number of cells that migrated in the absence of fMLP (Guilhem et al. 2013). This approach accounts for the chemotaxis or migration of neutrophils in the presence or absence of a chemotactic factor, respectively, and its results are identical to the percentage of neutrophils migrating towards fMLP.

Hertwig et al. (2016) proposed a more elaborate method of presenting the results of the Migratest® assay which is based on Δ migrating cells. This parameter is calculated as the difference between the ratio of the percentage of migrating neutrophils and the percentage of counting beads in an fMLP-stimulated sample and the ratio of the percentage of migrating neutrophils and the percentage of counting beads in a non-stimulated sample:

$$\Delta \text{ migrating cells (\%)} =$$

(% granulocytes in fMLP-stimulated sample)/(% counting beads in fMLP-stimulated sample) – (% granulocytes in non-stimulated sample)/(% counting beads non-stimulated sample)

In this study, the above method produced the most diverse results, and the differences in the chemotactic activity of granulocytes in experimental and control animals were more pronounced. The number of significant differences was higher, and Δ migrating cells (%) increased significantly in goats supplemented with HMB relative to non-supplemented animals throughout the entire experiment.

The Migratest® kit also supported the determination of the percentage of migrating neutrophils based on the expression of L-selectin (CD62L), a surface antigen in all types of leukocytes, including neutrophils, which plays an important role in cell recruitment and cell movement across the endothelium to bodily tissues in early stages of inflammation. In the analysed samples, the decrease in the expression of L-selectin (formerly referred to as LAM1 or LECAM-1) was correlated with the chemotactic activity of neutrophils. L-selectin was expressed in LECAM-1 control samples throughout the entire experiment. A significant decrease in the expression of L-selectin was observed on the surface of neutrophils in goats supplemented with HMB relative to non-supplemented animals (control).

The results of the current study were compared with published findings to reveal that the chemotactic index is the most effective approach to presenting the results of the Migratest® assay. The chemotactic index accounts for the chemotaxis or migration of neutrophils in the presence or absence of a chemotactic factor, respectively, as well as the percentage of granulocytes that migrate towards fMLP. Both presentation methods appear to produce identical results.

Acknowledgements

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