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RESEARCH ON THE IMMUNOMODULATORY EFFECT OF LEVAMISOLE IN SWINE

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Abstract

Immunomodulation is an important alternative in combating many diseases, being considered a potential weapon in the fight against pathological entities that cause major economic losses in pig herds. Levamisole, in addition to its anthelmintic effect, has also an immunostimulatory effect, for which it has also been used as a vaccine adjuvant. The aim of the research was to evaluate the non-specific immune response in modern techniques in pigs given levamisole. The results showed significant differences in the case of the ratio of lymphocyte subpopulations, there was an increasing trend in favor of T lymphocytes (with 10.44%), and the % of T lymphocytes blastic transformation (with 56%). Also, we observed a significant increase of the LTh/LTs ratio (with 65.92%) which supports the immunomodulatory potential of the levamisole and its involvement on the coordination of immune processes.

Key words*: immunomodulation, levamisole, lymphocytes, pigs.*

INTRODUCTION

Immunomodulation is an important alternative in combating many diseases and in the control of many infectious diseases in animals, the importance of the application of immunomodulatory therapies affecting the health of food, public health according to the common goal formulated based on the principles of "One Health" (Savu & Petcu, 2002; Goncearov et al., 2004; Petcu, 2006; Petcu et al., 2007).

The use of immunomodulatory preparations and microelements in pig herds, both in professional farming and in households, can increase the resistance of subjects to the action of pathogens, by positively regulating the duration and intensity of the immune response. Through the development and application of vaccines throughout history, many biological threats have been defeated, but techniques for enhancing the non-specific immune response have not received the same attention in the field of research. Non-specific immunomodulation becomes fundamental along with the principles of biosecurity in the case of the evolution of infectious pathologies that do not yet know a vaccine (Thacker, 2010; Marin et al., 2013).

Immunity, defined as resistance to disease, has as its main element a set of tissues, cells and molecules called the immune system, the corroborated action of its constituents giving rise to the reaction called the immune response (Fairbairn & Kapetanovic, 2011).

Immunomodulation is still an area of interest for veterinary medical research, being considered a potential weapon in the fight against pathological entities that cause major economic losses in pig herds. If immunosuppressive therapies are aimed at those situations in which it is necessary to resolve a harmful immune response. Immune stimulation may be a prophylactic or therapeutic alternative in case of infectious or parasitic pathologies.

Pharmaceutical products used in veterinary medicine for their immunostimulatory properties are either synthetic products or phytotherapeutic extracts or principles obtained from various microorganisms. The most common substances in this category are vaccine adjuvants intended to increase the effect of immunoprophylactic preparations in which they are included (Ioniță et al., 2014).

Levamisole, an imidazothiazole derivative, used primarily for its anthelmintic properties, has also an immunostimulatory effect, for which it has

also been used as a vaccine adjuvant (Galtier et al., 1983). After the administration, it resulted in better activation of regulatory T lymphocytes and intensified antibody production (Charerntantanakul & Roth, 2006). Moreover, the action of this molecule was correlated with a better maturation of dendritic cells, being stimulated the phagocytic functions of neutrophils and monocytes (Sajid et al., 2005).
The aim of the research was to evaluate the non-specific immune response with modern techniques in pigs given levamisole.

MATERIALS AND METHODS

In order to follow the objectives formulated in the paper, two groups of 10 animals each (control group and experimental group), of similar ages (6-7 months), belonging to a non-professional farm, were made. The selection of individuals was aimed at forming groups characterized by homogeneity in genetic characteristics, those related to age and maintenance conditions with full compliance with biosecurity conditions. Particular attention was paid to limiting the action of stressors, because stress hormones influence the dynamics of the leukocyte population (Ghiță et al., 2015). Additionally, the groups were homogeneous in terms of body mass because an excess of adipose tissue (by producing leptin) can change the ratio of lymphocyte populations (Ghiță et al., 2021).
The control group was subjected to treatment with saline, administered i.m., in a dose of 5 ml, 3 consecutive days.
The experimental group was treated with a product based on levamisole, respectively Levamisole 7.5%, solution for injection. It was administered i.m. at a dose of 2.5 mg/kg for 3 consecutive days.
Seven days after the last administration, blood samples were taken to check the immunomodulatory effect. Blood samples were collected on vacutainer devices by puncturing the auricular vein.
In order to make an objective assessment of the change in immune status following the administration of levamisole, a range of techniques characterized by high applicability were selected.
The methods used were: determination of WBC and Granulocytes/agranulocytes ratio (with IDEXX analyser), separation of total lymphocyte populations (using the separation technique with Ficoll medium), determination of LT and LB percentage (by EA rosetting technique), determination of ratio between LTh/LTs (by E rosetting technique), separation of populations from LT and LB (by separation technique on nylon fiber), determination of lymphoblastic transformation percentage of LT and LB (by lymphoblastic transformation technique (TTL) with mitogen Concanavalin A (Con A), variant based on the determination of the glucose consumption index in the medium of reaction), separation of the population from neutrophil polymorphonuclear (by the separation technique with the Dextran medium), determination of the locomotor capacity of the separated neutrophils (by the Boyden filter technique - directed migration density). It is noted that that the collected T and B lymphocyte layer isn't pure, containing 5% monocytes. The white layer that is formed on top of the erythrocyte layer contains most of the granulocytes. In case of separation of a bigger volume of blood (25 ml) the layer is denser, and it can be collected easier after 24 hours. In case that the entire deposit is collected, erythrocytes can be lysed by an osmotic shock with ammonium chloride. If the lysis is complete, a white deposit will form composed of polymorphonuclear granulocytes, and a red supernatant (haemoglobin and lysed erythrocyte membranes). The supernatant is removed, the remaining cell are washed three times in Hanks media, then the granulocytes are numbered, and they are resuspended with the desired concentration. The lymphocytes which form rosettes at higher temperatures and in the presence of lower erythrocyte concentration are called ***high affinity lymphocytes*** and ***high affinity E rosettes***, respectively. They represent 55-65% of the lymphocyte population, and apparently, they are part of the T helper lymphocyte subpopulation. If you subtract the number of high affinity E rosettes out of the total number of rosettes, you get the number of ***low affinity rosettes***, which apparently, are part of the T suppressor lymphocytes subpopulation.
The evaluation of blastization capacity by quantifying glucose consumption can be done by using multiple techniques. By heating glucose in an acidic solution, glucose forms with

ortho-toluidine a green compound with different colour intensity. With the help of a spectrophotometer you can measure the amount of used glucose after blastization. The technique is based on the phagocyte's capacity to cross a filtration system which contains chemoattractant substances. If the filtration system is transparent, the phagocytes which cross it will change the optical density of the filter, directly proportional with the number of migrating cells. First, FMLP solution is filtered through the Boyden filtration chamber. After filtration, the chemoattractant solution sticks to the filter. The phagocyte solution is inserted on the filter, after which the filter is incubated a few hours at 37°C. Phagocytes will cross the filter in the direction of the chemoattractant. The most widely used method consists on using transparent filters. Phagocytes get coloured while passing the filter, thus the filter will get coloured as well. The evaluation principle is based on quantifying the optical density of the filter (based on standard solution) which is proportional with the number of migrated cells.
Statistical analysis was performed using the t test (Student test).

RESULTS AND DISCUSSIONS

The obtained results will be presented in the form of a summary table and graphs, accompanied by comments.
Table 1 shows the analysed parameters for the two groups of animals.

Table 1. Values obtained for the two groups of animals (*p<0.01)

Analyzed parameter	Control group	Experimental group
WBC (10^9/l)	13.1	13.9
Granulocytes %	46	41.5
Agranulocytes%	54	58.5
T lymphocytes %	77.6	85.7*
B lymphocytes %	22.4	14.3
LTh/LTs ratio	2.5	3.9*
Lymphoblastic transformation LT%	40.2	66.7*
Lymphoblastic transformation LB %	44.1	44.9
Directed migration density (μ)	1674	1725

The results of the total leukocyte count (WBC) are shown in Table 1 and Figure 1.

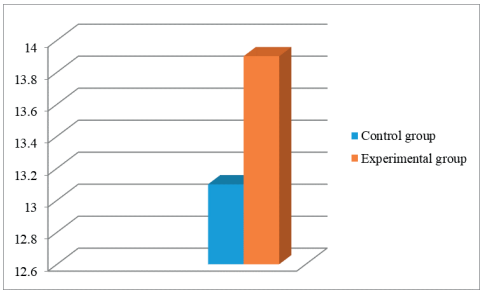

Figure 1. WBC values for both groups

Following the leukocyte count, an increase of 6.11% was observed in the experimental group, which reveals a small stimulatory effect of levamisole. This observation corresponds to data reported by other authors (Krakowski et al., 1999). Following the statistical analysis, it is observed that this increase is insignificant ($p < 0.05$). We mention that the values obtained fall within the physiological limits, in the case of both groups.
The results obtained regarding the granulocyte-agranulocyte ratio are presented in Table 1 and Figure 2.

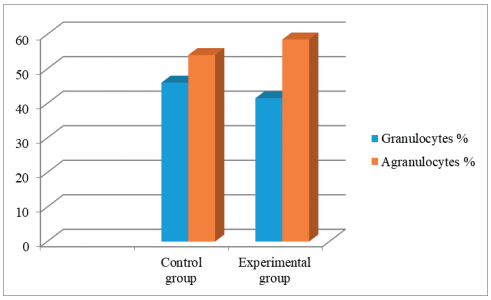

Figure 2. Granulocytes/agranulocytes ratio for both groups

Analysing the results presented in table 1 and figure 2, there is an insignificant increase ($p<0.05$) with 8.34% of the percentage of agranulocytes in the case of the experimental group. Corroborating with WBC, it can be concluded that the increase of this parameter is made on account of agranulocytes, respectively of lymphocytes, knowing that these are the most numerous agranulocytes. This observation can be explained by a mobilization of lymphocyte populations and subpopulations, in response to the stimulating effect of the administered substance (Valpotić et al., 2014).

Corroborating the data presented above, it is observed that 7 days after the end of treatment with Levamisole, the values regarding the number of total leukocytes and the granulocyte / agranulocyte ratio were kept within the physiological limits.
The T lymphocytes/B lymphocytes ratio results are shown in Table 1 and Figure 3.

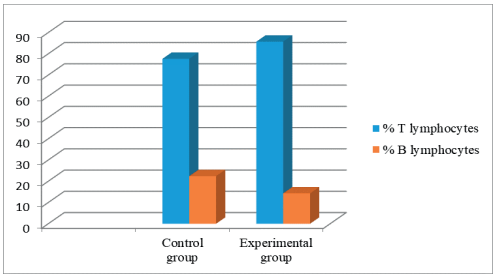

Figure 3. T lymphocytes/B lymphocytes ratio for both groups

Regarding the T lymphocytes / B lymphocytes ratio, resulting from the use of the EA rosetting technique, there is a 10.44% increase in the percentage of T lymphocytes. This significant increase ($p<0.01$) is due to the immunostimulatory effect of the administered product which stimulates the production of T lymphocytes, as also reported in the literature (Valpotić et al., 2009).
Regarding the calculation of lymphocyte subpopulation ratios, it showed an increasing trend in favor of T lymphocytes.
The obtained results regarding the LTh/LTs ratio using the EA rosetting technique are shown in Table 1 and Figure 4.

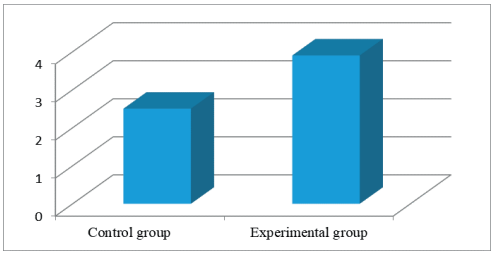

Figure 4. LTh/LTs ratio for both groups

Analysing the data presented above, a 56% increase in the LTh/LTs ratio is observed. This increase is significant ($p<0.01$) and is explained by the increase in LTh production following levamisole administration (Valpotić et al., 2009). In the case of our experiment, the hypothesis of an immunostimulatory effect obtained by administering levamisole is supported by the increase in the ratio of LTh/LTs. These cells represent in pigs over 90% of the total subpopulation of T lymphocytes.
The LTh/LTs ratio calculated 7 days after the last levamisole administration showed an increase in all 10 subjects in the experimental group. Thus, the dynamics of T lymphocyte populations with the increase of LTh weight, can support the immunomodulatory potential of the substance used and its effect on the coordination of immune processes (Suran et al., 2013, McHugh & Shevach, 2002).
The obtained results regarding the lymphoblastic transformation of T and B lymphocytes are presented in Table 1 and Figure 5.

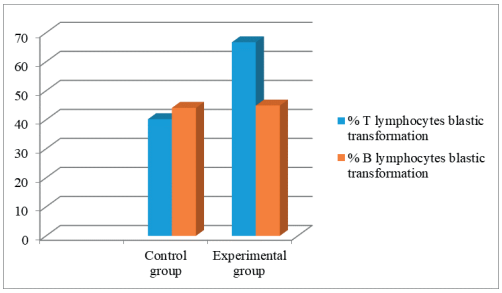

Figure 5. Percentage of T and B lymphocytes blastic transformation for both groups

Regarding the blastic transformation of T and B lymphocytes, there are increases in the percentage of blastic transformed lymphocytes for both categories of lymphocytes. But these increases are different. In the case of T lymphocytes, the increase has a value of 65.92% and is significant ($p<0.01$), while for B lymphocytes the increase is 1.82%, being insignificant ($p<0.05$). The mitogen used in the test was vegetal lectin recognized in the literature for having a specific receptor in both LT and LB. The difference observed in the case of lymphoblastic transformation of T lymphocytes is the notable change following the administration of levamisole.
The hypothesis of an immunostimulatory effect obtained by administering levamisole is supported by a significant increase in the lymphoblastic transformation index of T lymphocytes.

The blast index after stimulation with Con A changed very little in the case of B lymphocyte, the values obtained being close to the physiological ones. For T lymphocyte, however, there was an increase in this parameter in all pigs in the experimental group, with values between 62 and 68% (reference range after stimulation with Con A being 42-50%). These data may support the hypothesis that Levamisole has the ability to stimulate the cell-mediated immune response, the effect on the molecular component of immune reactions being uncertain.

The results for determining the migration capacity of neutrophils by determining the optical migration density directed by the Boyden filter are shown in Table 1 and Figure 6.

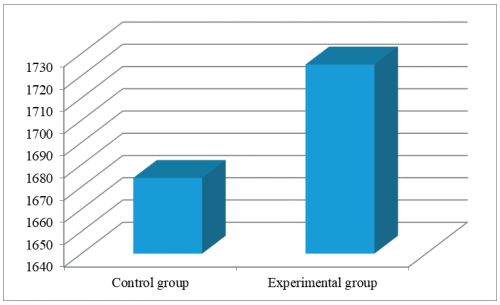

Figure 6. Directed migration density for both groups

From the presented data there is an insignificant increase ($p < 0.05$), with 3.05% in the case of the experimental group. This difference occurs when assessing the locomotion capacity of circulating phagocytes by the migration test under the agarose layer. The density of directed migration increases slightly, which shows that, in this case, levamisole may have caused an increase in phagocyte chemotaxis. Similar results were communicated by other authors (Gâjâilă et al., 2016).

When evaluating the locomotor functions of circulating phagocytes, results have been reported that cannot fully support the stimulatory effect on the chemotaxis of these cells. The optical density of directed migration increased significantly (by 54.4%) only in the case of a single individual, which even decreased by 1.6% according to data obtained from another subject in the same group. A possible bacterial infection without clinical expression in the animal in which the increase of the migration index was found could be a plausible explanation (Gâjâilă et al., 2016). The optical density values, regardless of the percentage change suffered, were kept within the reference range: 1200-1900µ. These aspects do not allow us to establish the effect of levamisole on phagocytic functions.

CONCLUSIONS

Levamisole altered the ratio between T lymphocytes and B lymphocytes in favor of T lymphocytes, which demonstrates an activation of the cell-mediated immune response.

Levamisole has been implicated in the mechanisms of coordination of immune processes, increasing the ratio of LTh/LTs to values exceeding the physiological threshold.

The action of levamisole on B lymphocytes is insignificant because the population of these lymphocytes has not increased in number and percentage.

The test used to evaluate the phagocytic functions demonstrated its limitations and did not allow the signaling of a direct way to stimulate the chemotaxis of the tested phagocytes.

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