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Original Research Paper

LEVAMISOLE INCREASES LEUKOCYTE COUNT AND IMMUNOGLOBULIN LEVELS IN YOUNG BOARS

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Summary: Levamisole has been used as an immunomodulator in the treatment of some diseases and conditions include parasitic invasions and immunocompromised conditions in domestic animals. The aim of the study is usage of levamisole to modulate the immune responsiveness in young boars in condition of intensive exploitation like handling using restraint. Therefore, hematological and immunological variables were assessed in Swedish Landrace boars treated with levamisole for three consecutive days. Levamisole treatment effected a significant increase in total leukocyte count immediately upon the last dosage (day 3, $p<0.01$), which persisted to days 4, 5 and 6 ($p<0.01$ to $p<0.001$). Also, the percentage of neutrophilic granulocytes increased significantly on days 5, 6 and 7 ($p<0.001$ and $p<0.0001$), whereas the percentage of eosinophil cells decreased significantly on days 7 and 11 after levamisole administration ($p<0.01$ and $p<0.001$). Lymphocyte, basophil and monocyte counts were unaffected by levamisole treatment. Serum IgG concentration was significantly elevated on days 5, 6, 7, 11 and 14 ($p<0.01$ to $p<0.001$, respectively), whereas IgA concentration showed a significant increase only on day 14 ($p<0.01$) after levamisole administration. Levamisole treatment had no effect on serum levels of IgM. Cortisol levels remain unaffected throughout the period of observation. Levamisole treatment increased nonspecific cellular and humoral immunity in boars.

Key words: levamisole; leukocytes; immunoglobulins; boars

Introduction

Both veterinary and human medicine need a drug that would influence immune reactivity in conditions such as inflammation, infections, malignant diseases, and even in the prevention of stress effect on the immune function. Many studies have shown that immunomodulatory substances have a limited mode of action (2, 9).

Levamisole (*levo* isomer of 2,3,5,6-tetrahydro-6-phenylimidazo (2,1-*b*) thiazole) is primarily used as a broad-spectrum anthelmintic. However, a number of clinical and experimental trials have shown that levamisole may be beneficial as an immunomodulator in the treatment of some diseases (8, 21, 25), yet only partially meeting the expectations, as its therapeutic efficacy depends on several factors such as the dose administered, timing, and immune status of animal or human patients, which is of utmost importance. The drug exerts stimulatory action on the immune and inflammatory functions of leukocytes through stimulation of lymphocyte proliferation, cytotoxicity, chemotaxis, spontaneous motility and phagocytosis (25). Previous investigations have suggested that levamisole changes balance between the cAMP and cGMP cyclic nucleotides by increasing intracellular levels of cGMP in leukocytes (12, 21).

The aim of the study is usage of levamisole to assess the immunomodulatory effect in boars in condition of intensive exploitation like handling using restraint. Therefore, levamisole is administered in a recommended immunostimulatory dose of 2-3 mg/kg body weight to young boars for three days. Changes in total and differential leukocyte count and humoral nonspecific (immunoglobulins) immunity were monitored for more than two weeks

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after levamisole treatment. Cortisol concentrations were simultaneously observed because the handling procedure used in blood sampling may stress boars.

Materials and methods

Boars

Fourteen boars (Swedish Landrace) aged about 7 months and weighing between 105 and 125 kg were used. The boars were housed in individual pens in a stable at a temperature of 12-18 °C. Boars were fed individually 3 kg of standard corn-soy ration per day (components: starch 37.96%, sugar 3.02%, crude protein 16.99%, crude fiber 5.26%, crude fat 5.39%), with free access to water.

On each of the three experimental days, levamisole and saline were administered before 10.00 a.m. Both groups of boars were handled using restraint with a snare in the procedures of levamisole and saline administration and blood collection. The experimental group of seven boars were intramuscularly primed with levamisole (Nilverm®, Pliva, Zagreb, Croatia; the preparation contains 75 mg levamisole hydrochloride/mL) at the immunostimulatory dose of 2.5 mg/kg body weight on three consecutive days. The control group of boars (n=7) were intramuscularly administered 1 mL of sterile 0.9% saline on three consecutive days as a placebo.

Blood sampling

Blood samples from the treated and control boars were obtained between 8.00 and 10.00 a.m. on day 1 before the first levamisole injection, on day 3 after the third levamisole injection, then on days 4, 5, 6, 7, 11, 14, 18 and 22 after the last drug dosage. Briefly, the boars were restrained with a snare and blood was collected from jugular vein by sterile syringe (Becton Dickinson S.A., Fraga Huesca, Spain). Blood samples were immediately transferred to glass tubes containing EDTA solution for plasma samples (K3E 15% DB Vacutainer®, Preanalytical Solutions Belliver Industrial Estate, Plymouth, UK) and tubes for serum samples (SST, DB Vacutainer®, Preanalytical Solutions Belliver Industrial Estate, Plymouth, UK). Blood samples were centrifuged at 750 x g for 10 min, and then serum was separated and stored at -20 °C until analysis.

Hormone assays

Cortisol concentrations were determined by radioimmunoassay using commercially available RIA Coat-A-Count Kit (Diagnostics Products Corp., Los Angeles, USA) according to the manufacturer's instructions. Samples were quantified with average intra- and inter-assay coefficients of variation (n=5) of 7.5% and 12.0%, respectively. The assay sensitivity was 0.1 nmol/L.

Total and differential leukocyte counts

Total leukocyte counts were determined on a Baker System 9120 CP cell counter (Serono-Baker Diagnostics Inc., Allentown, PA, USA). For leukocyte differentiation, blood smears were stained with May-Grünwald-Giemsa. The percentage of neutrophils, lymphocytes, eosinophils, basophils and monocytes was counted by use of a microscope (Carl Zeiss, GF-Planchomat, Jena, Germany) at x100 magnification with oil immersion lens. A total of 100 leukocytes were counted from each slide, classified as different cell types, and expressed in percentage.

Immunologic parameters

Immunoglobulins were tested by radial immunodiffusion method (20). Test plates for determination of IgG were prepared by dilution of 2 g of agarose in 100 mL of barbiturate buffered saline (0.1 M) with the addition of anti-pig IgG antiserum (1:10) and 0.1% sodium azide. Five microlitres of reference standard solutions of IgG and diluted serum samples (1:20) were pipetted to a separately identified well of test plates. The plate was securely covered and incubated for 48 to 72 hours at room temperature. After incubation, the plates were removed and placed over a source of illumination to clearly see precipitation rings. The external diameters of the rings were measured to the nearest 0.1 mm by using an ocular scale. A reference curve was plotted using the diameters measured from standard solutions. From the reference curve, the IgG concentration of each diluted test sample was calculated by multiplying the concentration read from the curve by the dilution factor to obtain the actual concentration. Intra- and inter-assay coefficients of variation were 3.5% and 5.4%, respectively .

The concentrations of immunoglobulins A and M were determined by use of commercially available pig IgA and IgM VET-RID kits (Bethyl Laboratories,

Inc., Montgomery, Texas, USA). Intra- and inter-assay coefficients of variation (n=5) were 1.8% and 2.8% for IgA, and 0.5% and 1.2% for IgM, respectively.

Statistical analysis

Data were analyzed by Statistica® software ('99 Edition, Copyright 1984-1999, StatSoft®, Inc., Tulsa, USA). Results were expressed as mean ± SEM. Differences in total and differential leukocyte counts and humoral immune parameters in the treated and control group were examined using analysis of variance. To evaluate differences in the means between control and treated groups of animals at specific time points we used *t*-test for independent samples. The differences between values were considered significant at $p \leq 0.05$.

Results

Cortisol concentrations

The mean serum cortisol response in the groups of boars is shown in Figure 1. The administration of saline and levamisole had no effect on cortisol concentrations throughout the period of observation.

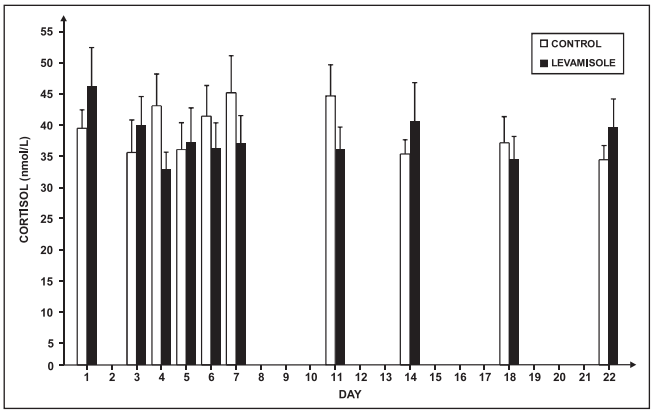

Figure 1: Serum cortisol concentration (mean ± SEM; nmol/L) in boars treated with levamisole (n=7) and saline (n=7)

Alterations in total and differential leukocyte counts

Changes in total and differential leukocyte counts in the experimental and control groups of animals after repeated levamisole and saline challenge are shown in Figures 2 to 4. The levamisole treated animals showed a significant increase in total leukocyte count immediately after the last levamisole dosage on day 3 ($p<0.01$), which persisted on days 4, 5 and 6 ($p<0.01$ to $p<0.001$, respectively) (Fig. 2). There were no significant differences in lymphocyte count between levamisole treated (49.7 ± 1.02 to 55.3 ± 4.85 %) and control (49.8 ± 3.02 to 53.8 ± 1.98 %) group of boars throughout the study period. Basophile and monocyte counts were not influenced by levamisole application either, so there were no differences between levamisole treated (basophiles: 0.57 ± 0.297 to 1.14 ± 0.594 %; monocytes: 0.58 ± 0.202 to 1.2 ± 0.632 %) and control animals (basophiles: 0.50 ± 0.341 to 1.20 ± 0.508 %; monocytes: 0.75 ± 0.387 to 1.0 ± 0.516 %). However, levamisole significantly increased the percentage of neutrophilic granulocytes on days 5, 6 and 7 after treatment ($p<0.001$ and $p<0.001$) (Fig. 3). The levamisole treated animals showed a significantly lower percentage of eosinophil cells on days 7 and 11 after the last dosage ($p<0.01$ and $p<0.001$, respectively) (Fig. 4).

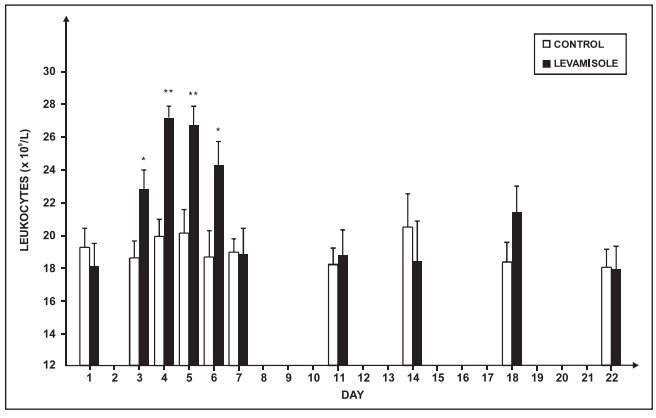

Figure 2: Total leukocyte count (mean ± SEM; $x10^9$/L) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$; ** $p<0.001$

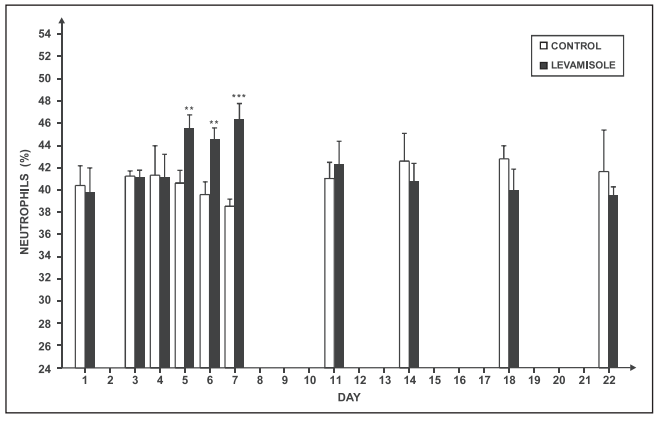

Figure 3: Neutrophil percentage (mean ± SEM; %) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: ** $p<0.001$; **** $p<0.0001$

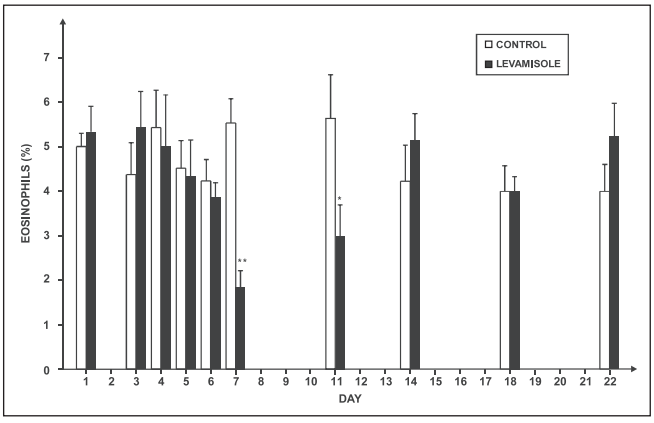

Figure 4: Eosinophil percentage (mean ± SEM; %) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$; ** $p<0.001$

Immunologic measures

The mean serum concentrations of immunoglobulin IgG, IgA and IgM levels after injections of levamisole and saline are shown in Figures 5, 6 and 7. Serum IgG concentration was significantly elevated on days 5, 6, 7, 11 and 14 ($p<0.01$ to $p<0.001$, respectively) (Fig. 5), whereas IgA concentration was significantly increased on day 14 ($p<0.01$) after levamisole administration (Fig. 6). Levamisole treatment had no effect on serum levels of IgM (Fig. 7).

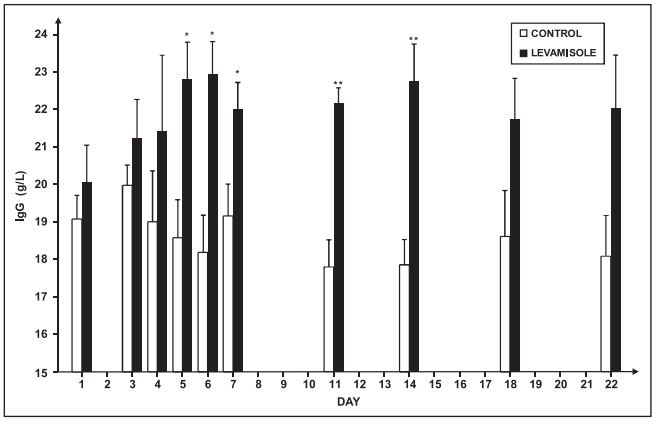

Figure 5: Serum IgG concentration (mean ± SEM; g/L) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$; ** $p<0.001$

Discussion

Different situations in modern farming are considered stressful to pigs, e.g., physical restraint and type of housing (10, 16). In this study, handling procedure by snare restraint on saline and levamisole

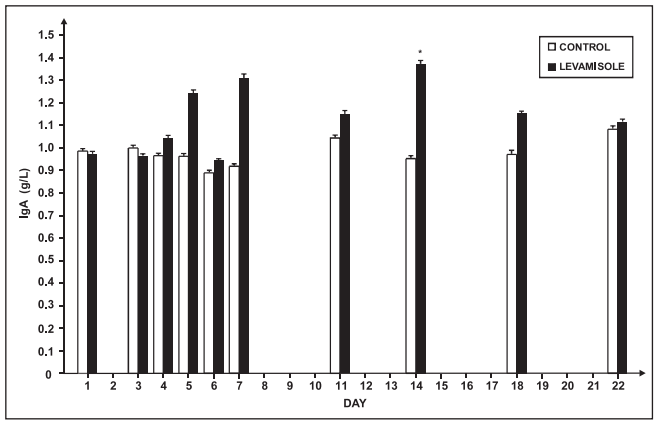

Figure 6: Serum IgA concentration (mean ± SEM; g/L) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$

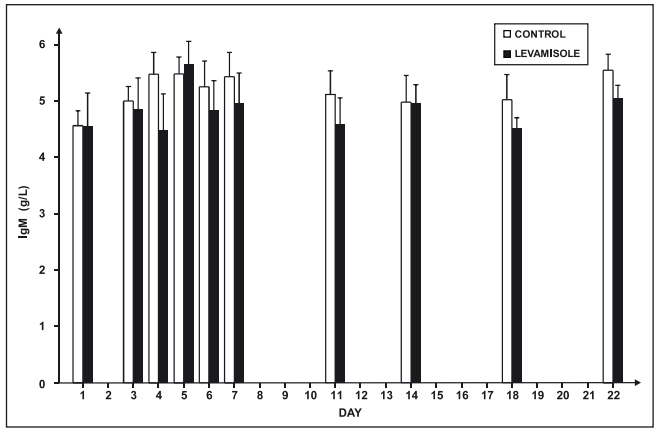

Figure 7: Serum IgM concentration (mean ± SEM; g/L) in boars treated with either levamisole (n=7) or saline (n=7)

administration as well as on blood sampling did not produce a stress condition in boars. Cortisol response remained stable throughout the experimental period in both groups of animals.

In contrast to higher anthelmintic dosage, levamisole was demonstrated to restore immune function in a dose of 2-3 mg/kg body weight, and has been recommended to be administered for three consecutive days or once a week as a single dose. Previous studies of levamisole as an immunomodulatory drug have suggested an immunity improvement to occur in cells with impaired function (8, 25). Therefore, the results and suggestions of different experiments have been rather contradictory and inconclusive (2). However, in the present study repeat levamisole injection changed total and differential leukocyte count in young healthy boars. Levamisole induced a significant increase in total leukocyte count immediately after the last dosage

(day 3), which persisted on days 4, 5 and 6 after treatment. Also, levamisole significantly elevated the percentage of neutrophilic granulocytes on days 5, 6 and 7, whereas the percentage of eosinophil cells significantly decreased on days 7 and 11 after application. On the other hand, lymphocyte, basophile and monocyte counts remained unaffected by levamisole treatment throughout the study period. In previous study is determined that levamisole treatment of boars with 2.5 mg/kg for three days increase leukocytes and monocytes 7 weeks after test termination (23).

The use of levamisole in the immunostimulatory dose of 2.5 mg/kg body weight in weaned piglets infected with enterotoxigenic *Escherichia coli* strain before vaccination suggested that levamisole restored leukocyte count to normal rather than stimulating it above the normal level (3). Also, the phagocytic function in vaccinated pigs was impaired by their pretreatment with levamisole. The administration of levamisole in the same dose (2.5 mg/kg body weight) enhanced lymphocyte proliferation in artificially reared pigs (13). It has also been reported that levamisole can enhance immune responses to viral antigens and reduce the incidence of disease (1, 11). Recent studies suggested that levamisole has a tendency for selective induction of protective mucosal cell-mediated immune response in pigs vaccinated against *Escherichia coli* (3, 5, 6).

The effect of levamisole on immunoglobulin synthesis in animals or humans is not completely understood, and it seems to primarily depend on the dosage used and duration of treatment (2, 22, 25). In our study, levamisole treatment of boars for three consecutive days in a dose of 2.5 mg/kg body weight led to a significant increase in serum IgG concentration on days 5, 6, 7, 11 and 14 after treatment. At the same time, serum IgA concentration was significantly increased only on day 14, whereas serum IgM levels were unaffected by levamisole treatment. Levamisole was demonstrated to increase serum IgG levels in foals from mares treated with levamisole (18). Therefore, the authors assumed that levamisole could protect foals against infectious disease during the postnatal period. Also, the colostrum of mares stimulated with levamisole was characterized by an increase in IgG, however, IgA and IgM classes remained unaffected.

Levamisole has a broad, well-defined activity on T lymphocytes. It has also been postulated that any effect on B lymphocyte function may probably occur indirectly through stimulation of T lymphocytes and macrophages rather than B lymphocyte cells themselves (8, 25). A recent study has shown that levamisole has a highly selective activity on immune response by resetting the immune balance of T lymphocyte subsets towards Th1 response (24, 26). On the other hand it has been shown that levamisole enhance both Th1 and Th2 type response (17). Levamisole has been shown to increase serum antibody titters after immunization, the number of leucocytes, phagocyte activities, the expression of cytokines by monocyte/macrophages, lymphocyte proliferation and antitumor responses (4, 7, 14, 15, 19, 23, 27).

In conclusion, the present study showed that administration of levamisole in recommended immunostimulatory dose for three consecutive days stimulated elevation of total leukocyte count and percentage of neutrophil cells in healthy boars. Also, levamisole induced an increase in serum IgG concentration that persisted for ten days after levamisole application. These observations suggest that levamisole also enhances immune response in cells with normal function and may be effective as a protective drug against diseases or stressful situations.

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LEVAMISOLE ZVIŠUJE ŠTEVILO LEVKOCITOV IN RAVEN IMUNOGLOBULINOV PRI MLADIH PRAŠIČIH

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Povzetek: Levamisole je v uporabi kot imunomodulator za zdravljenje nekaterih bolezni in stanj, kot so vdor zajedavcev in stanje imunske zavrtosti pri domačih živalih. Cilj študije je z uporabo levamisola uravnati imunsko odzivnost pri mladih prašičih v stanju intenzivnega izkoriščanja, kot je ravnanje s prašiči z uporabo prisile. V ta namen so bili ocenjeni hematološki in imunološki parametri pri prašičih švedske deželne pasme, ki so jih tri zaporedne dni zdravili z levamisolom. Zdravljenje z levamisolom je značilno vplivalo na povišanje skupnega števila levkocitov takoj po zadnjem odmerku (3. dan, $p<0.01$) in je trajalo do 4., 5. in 6. dneva ($p<0.01$ do $p<0.001$). Prav tako se je značilno povišal odstotek nevtrofilnih granulocitov 5., 6. in 7. dan ($p<0.001$ in $p<0.0001$), medtem ko se je odstotek eozinofilnih granulocitov značilno znižal 7. in 11. dan po uporabi levamisola ($p<0.01$ in $p<0.001$). Število limfocitov, bazofilnih granulocitov in monocitov je ostalo po zdravljenju z levamisolom nespremenjeno. Serumska koncentracija IgG je bila značilno povišana 5., 6., 7., 11. in 14. dan ($p<0.01$ do $p<0.001$), medtem ko je bila koncentracija IgA značilno povišana le 14. dan ($p<0.01$) po uporabi levamisola. Zdravljenje z levamisolom ni vplivalo na raven IgM v serumu. Raven kortizola je ostala v času opazovanja nespremenjena. Zdravljenje z levamisolom je pri prašičih povišalo nespecifično celično in humoralno imunost.

Ključne besede: levamisole; levkociti; imunoglobulini; prašiči