

The influence of levamisole on cortisol concentration and peripheral blood in artificially stressed pigs

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BILANDŽIĆ, N., B. ŠIMIĆ, S. TERZIĆ, M. ŽURIĆ: The influence of levamisole on cortisol concentration and peripheral blood in artificially stressed pigs. Vet. arhiv 80, 477-489, 2010.

ABSTRACT

The objective of the study was to evaluate the influence of levamisole (LEV) application in pigs stressed by adrenocorticotropic hormone (ACTH) injection on the total and differential leukocyte count, blood erythrocyte count, haemoglobin concentration and cortisol level during and for 16 days after the treatment. Swedish Landrace boars aged 6 to 7 months were divided into three groups. Group 1 received levamisole 2.5 mg/kg/body mass/day intramuscularly over 3 days and ACTH 10 µg/kg/body mass/day intravenously for 3 days. Group 2 received ACTH 10 µg/kg/body mass/day intravenously for the next 3 days and group 3 received saline intramuscularly for 6 days (control group). Cortisol concentration was increased in both ACTH treated groups during all three days of administration and the day after the last ACTH treatment. A significantly increase in total leukocyte count and a decrease in lymphocyte percentages was recorded during ACTH treatment. Use of levamisole before stress induction caused an increase in total leukocyte count in the 16 day period after cessation of ACTH treatment and also a lymphocyte increase in stressed animals on the first day of ACTH injection. Pigs that received levamisole and ACTH did not show eosinophilia in contrast to pigs that received ACTH only. In both groups of stressed animals, an elevated percentage of neutrophilic granulocytes was recorded on days 2 and 3 of ACTH treatment. However, administration of levamisole led to faster normalization of neutrophils and prevented neutropenia one week after termination of stress. According to the data presented, levamisole can influence pig immunity during and after stress induction by ACTH administration.

Key words: levamisole, adrenocorticotropic hormone, stress, immunomodulation, boars, leukocytes

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Introduction

Different stress situations in animals may suppress the non-specific defence mechanism and cause an increased susceptibility to disease and infection (BLECHA, 1988; GRIFFIN, 1989). Previous reports have shown that stress can affect multiple aspects of immune functions in pigs (MORROW-TESCH et al., 1994; DE GROOT et al., 2001). In intensive husbandry systems, stressful events (weaning, bacterial infections or new social environments) may change their behaviour and physiology, and also decrease cellular and humoral immune response (MORROW-TESCH and ANDERSSON, 1994; NAGY and FEKETE, 1999). Also, prenatal stress affects the humoral and cellular immune responses of neonates and it is able to alter the postnatal response of the immune system to stress (TUCHSCHERER et al., 2002).

Modern veterinary and human medicine uses drugs to modify immune response in abnormal immune function conditions and in managing disease. Levamisole, a substituted thiazole, is a drug that has been intensively studied as an immunomodulating agent in humans and animals (SYMOENS and ROSENTHAL, 1977). Primarily, levamisole is a potent broad-spectrum anthelmintic, which is active against most pathologic nematodes in animals and man. Contrary to the higher anthelmintic dosage, levamisole modulates immune function at a dose of 2-3 mg/kg body mass (RENOUX and RENOUX, 1974; BRUNNER and MUSCOPLAT, 1980). Levamisole restores immune response effectively in intermittent rather than continuous treatment (SYMOENS and ROSENTHAL, 1977) and within three days or as a single dose once a week (BRUNNER and MUSCOPLAT, 1980). The primary action of levamisole seems to be a cell-mediated immune response through enhancing immune response with special influence on cells with impaired function (BRUNNER and MUSCOPLAT, 1980; MULCAHY and QUINN, 1986). Levamisole has been shown to increase serum antibody titers after immunization, the number of leucocytes, phagocyte activities, the expression of cytokines by monocyte/macrophages, lymphocyte proliferation and antitumor responses (SYMOENS and ROSENTHAL, 1977; TEMPERO et al., 1995; SZETO et al., 2000; BOŽIĆ et al., 2002, 2006; JANJATOVIĆ et al., 2008).

The present study investigated the effect of levamisole (LEV) in animals stressed for 3 days by adrenocorticotrophic hormone (ACTH) injection into cortisol concentration. The stressed animals received levamisole in an immunomodulating dose on 3 consecutive days prior to ACTH induction. Erythrocyte count, haemoglobin concentration, total leukocyte count and differential leukocyte count (neutrophils, lymphocytes and eosinophils) were determined in the blood of the treated pigs to assess the effect of levamisole on peripheral blood changes in stressed pigs.

Materials and methods

Animals. A total of 18 Swedish Landrace boars aged 6 to 7 months and weighing between 105 and 135 kg were used in the study. The boars were kept in individual pens at

a stable temperature of 10-17 °C. Water was available *ad libitum*. The pigs were fed 3 kg of standard corn-soy ration *per day* containing crude protein 16.99%, crude fiber 5.26%, crude fat 5.39%, starch 37.96%, sugar 3.02% and ashes 5.65%.

The boars were randomly assigned to three experimental groups. All pigs were handled using restraint with a snare in the procedure for administering levamisole, ACTH and saline (at 10.00 a.m. each of the six experimental days) as well as for blood collection. Pigs from the first group (LEV+ACTH, n = 6) received intramuscularly on days 1, 2 and 3 2.5 mg/kg body mass of levamisole (Nilverm®, Veterina d.o.o. Zagreb, Croatia, 75 mg levamisole hydrochloride/mL). Then, the animals were injected with ACTH (ACTH, from porcine pituitary, 80 IU/mg, Sigma-Aldrich Co., St. Louis, USA) in a dose of 10 µg/kg body mass for 3 days (days 4, 5 and 6) into the ear vein. Boars from the second group (ACTH, n = 6) received intramuscularly 1 mL of sterile 0.9% saline on days 1, 2 and 3, then, the animals were injected with 10 µg/kg body mass of ACTH on days 4, 5 and 6 into the ear vein. Control pigs (n = 6) received 1 mL of sterile 0.9% saline intramuscularly daily.

Blood sampling. All boars were frequently handled and habituated to the blood collection procedure to be performed *via* the jugular vein on the first and third day of the experiment before the first and after the third levamisole administration. On each day of ACTH treatment (days 4, 5 and 6) blood samples were collected 90 min after drug administration (ACTH or saline). Furthermore, blood samples were collected on days 7, 11, 14, 18 and 22 after the last drug dosage. Blood samples were obtained using 20 mL sterile syringes (Becton Dickinson S.A., Fraga, Spain) and 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 g for 10 min, when the serum was separated and stored at -20 °C until analysis.

Hormone assays. Cortisol concentrations were determined by radioimmunoassay using a 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 of 7.5% and 12.0%, respectively. The assay sensitivity was 0.1 nmol/L.

Haematological parameters. Blood erythrocyte count, haemoglobin concentrations and total leukocyte count (WBC) were determined on a Baker System 9120 CP cell counter (Serono-Baker Diagnostics Inc., Pennsylvania, USA). For leucocyte differentiation, blood smears were stained with May-Grünwald-Giemsa. The percentage of neutrophils, lymphocytes and eosinophils was counted by use of a microscope (Carl Zeiss, GF-Planchomat, Jena), ×100 magnification with oil immersion lens. A total of 100 leucocytes

were counted from each slide and classified as different cell types, and then expressed as percentage.

Statistical analysis. Statistical analysis was performed using the Statistica® software package (99th Edition, Copyright 1984-1999, StatSoft®, Inc., Tulsa, USA). All data were expressed as mean \pm SEM. Differences in studied immune and hormonal parameters in treated (ACTH and LEV+ACTH) and control groups were examined using analysis of variance (ANOVA). To evaluate the differences in means between groups of animals at specific time points we used the *t*-test for independent samples. Results were considered significantly different at $P < 0.05$.

Results

Elevation of cortisol levels induced by ACTH treatment. The mean serum cortisol response in the experimental groups of boars is shown in Fig. 1. The administration of 10 $\mu\text{g}/\text{kg}$ body mass of ACTH in the experimental groups (ACTH and LEV+ACTH) induced an increase in serum cortisol concentration after 90 minutes on all three days of ACTH administration. The administration of saline and levamisole had no effect on cortisol concentrations.

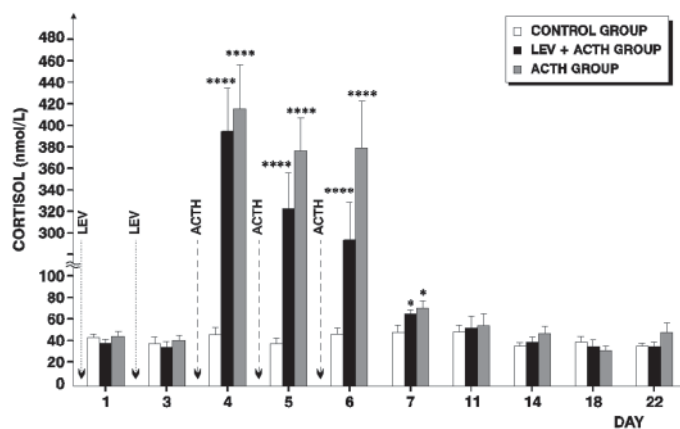


Fig. 1. Cortisol (mean \pm SEM; nmol/L) response to ACTH administration in boars treated with ACTH (10 $\mu\text{g}/\text{kg}$ / body mass/day intravenously for 3 days; ACTH group, $n = 6$), levamisole (2.5 mg/kg/ body mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, $n = 6$), and saline (1 mL intramuscularly for 6 days; control group, $n = 6$). Significant differences between groups: LEV+ACTH vs. control and ACTH vs. control (* $P < 0.05$; **** $P < 0.0001$).

In both ACTH treated groups of boars increase in cortisol concentrations were highly significant as compared with cortisol concentration after saline infusion on each treatment

day ($P < 0.0001$). Also, the concentrations of cortisol were significantly higher in both ACTH treated groups 24 hours after the last ACTH dosage in comparison with control boars ($P < 0.05$). Thereafter, the concentration of cortisol returned to normal levels on days 11, 14, 18 and 22 after the last drug dosage. There was no significant difference in cortisol concentration between the two groups treated with ACTH.

Peripheral blood changes. Blood erythrocyte count (Fig. 2) and haemoglobin concentration (Fig. 3) were not influenced by levamisole and ACTH treatment.

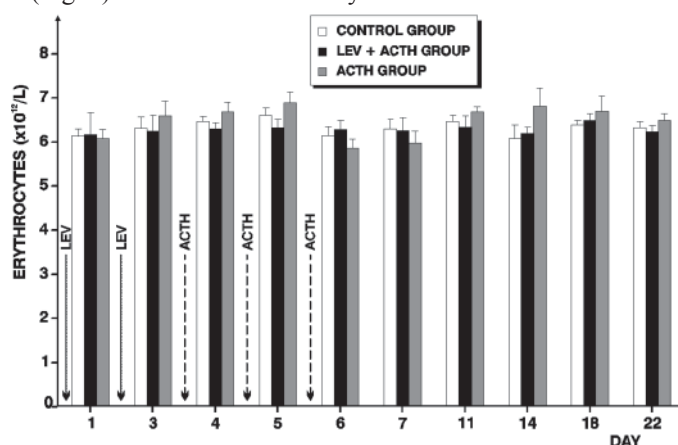


Fig. 2. Blood erythrocyte count (mean \pm SEM; $\times 10^{12}/L$) in boars treated with ACTH (10 $\mu g/kg$ / body mass/day intravenously for 3 days; ACTH group, $n = 6$), levamisole (2.5 mg/kg/ body mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, $n = 6$), and saline (1 mL intramuscularly for 6 days; control group, $n = 6$).

Changes in total and differential leukocyte counts in the experimental and control groups of animals during and after repeated ACTH, levamisole/ACTH and saline challenge are shown in Figures 4-7. Simultaneously with the increase of cortisol concentration, in ACTH and LEV+ACTH treated animals there was a significant increase in total leukocyte count ($P < 0.05$ to $P < 0.0001$, respectively) in comparison with control levels (Fig. 4). On the other hand, the use of levamisole before stress induction (LEV+ACTH) led to a significant increase in total leukocyte count vs. control values ($P < 0.05$ to $P < 0.0001$) for a period of 16 days after the cessation of ACTH treatment. However, the leukocyte count in the ACTH treated group remained unchanged on the 7th, 11th, 14th, 18th and 22nd days after cessation of treatment. Furthermore, total leukocyte count in the LEV+ACTH group was also significantly higher in comparison with ACTH treated animals in the post-stress period 7th, 11th, 14th, 18th and 22nd day ($P < 0.05$ to $P < 0.0001$).

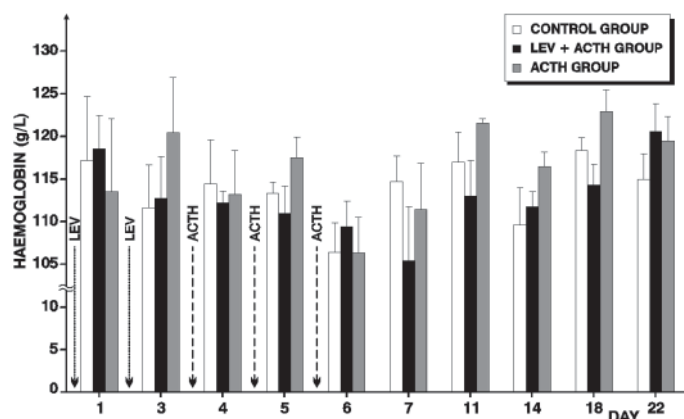


Fig. 3. Haemoglobin concentrations (mean \pm SEM; g/L) in boars treated with ACTH (10 μ g/kg/ body mass/day intravenously for 3 days; ACTH group, n = 6), levamisole (2.5 mg/kg/ body mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, n = 6), and saline (1 mL intramuscularly for 6 days; control group, n = 6).

However, the use of ACTH treatment induced a significant decrease ($P < 0.01$ to $P < 0.0001$, respectively) in the lymphocyte percentage during ACTH administration (Fig. 5). Lymphopenia resolved upon discontinuation of ACTH treatment. On the other hand, the use of levamisole before stress induction (LEV+ACTH) led to an increase of lymphocyte percentage in stressed animals, especially on the first day of ACTH administration, when the LEV+ACTH group of animals had a significantly higher lymphocyte percentage ($P < 0.001$) vs. the ACTH group. The stress induced by ACTH injection significantly elevated the percentage of neutrophilic granulocytes in the ACTH ($P < 0.0001$) and LEV+ACTH ($P < 0.01$) groups of boars on days 2 and 3 of ACTH treatment (Fig. 6). On day 11 in the week after the treatment, the percentage of neutrophils significantly decreased ($P < 0.05$) in ACTH treated boars. Simultaneously, the neutrophil count in LEV+ACTH boars was significantly higher ($P < 0.05$ to $P < 0.01$, respectively) as compared with ACTH boars on day 3 of ACTH treatment and on days 11 and 14 after the treatment.

The percentage of eosinophils remained unchanged during stress treatment in pigs that received levamisole before ACTH application (Fig. 7), in contrast to ACTH treated pigs who showed an eosinophil count elevation on days 1 and 2 of ACTH administration to control and LEV+ACTH groups ($P < 0.01$ to $P < 0.0001$, respectively). On the day after treatment discontinuation, the percentage of eosinophils in LEV+ACTH treated boars decreased significantly vs. control ($P < 0.001$) and ACTH ($P < 0.01$) groups.

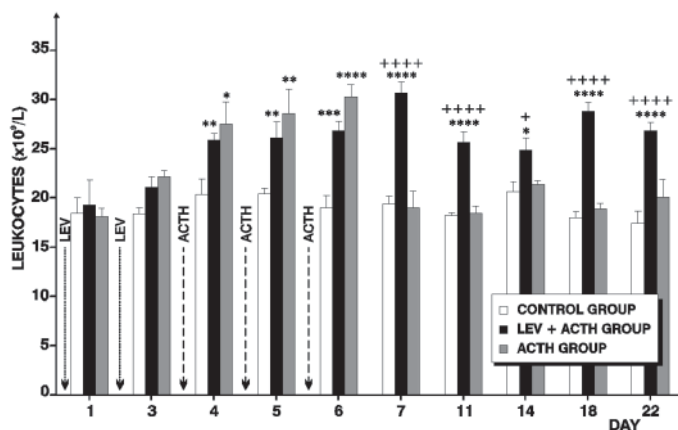


Fig. 4. Total leukocyte count (mean \pm SEM; $\times 10^9/L$) in boars treated with ACTH (10 $\mu g/kg$ / body mass/day intravenously for 3 days; ACTH group, n = 6), levamisole (2.5 mg/kg/ mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, n = 6), and saline (1 mL intramuscularly for 6 days; control group, n = 6). Significant differences between groups: LEV+ACTH vs. control and ACTH vs. control (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001); LEV+ACTH vs. ACTH (+P<0.05; ++P<0.01; +++P<0.0001).

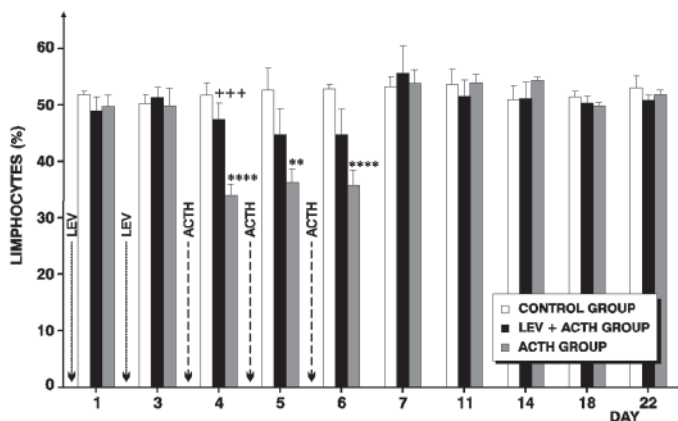


Fig. 5. Lymphocytes (mean \pm SEM; %) in blood of boars treated with ACTH (10 $\mu g/kg$ / body mass/day intravenously for 3 days; ACTH group, n = 6), levamisole (2.5 mg/kg/ body mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, n = 6), and saline (1 mL intramuscularly for 6 days; control group, n = 6). Significant differences between groups: ACTH vs. control (**P<0.01; ****P<0.0001); LEV+ACTH vs. ACTH (+++P<0.001).

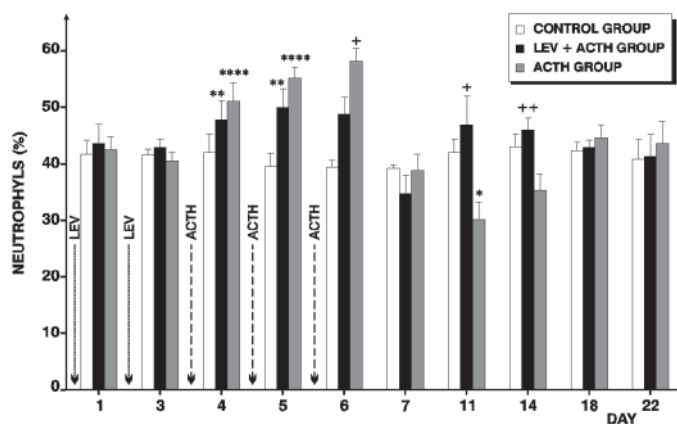


Fig. 6. Neutrophils (mean \pm SEM; %) in blood of boars treated with ACTH (10 μ g/kg/ body mass/day intravenously for 3 days; ACTH group, n = 6), levamisole (2.5 mg/kg/ body mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, n = 6), and saline (1 mL intramuscularly for 6 days; control group, n = 6). Significant differences between groups: LEV+ACTH vs. control and ACTH vs. control (* P <0.05; ** P <0.01; **** P <0.0001); LEV+ACTH vs. ACTH (+ P <0.05; ++ P <0.01).

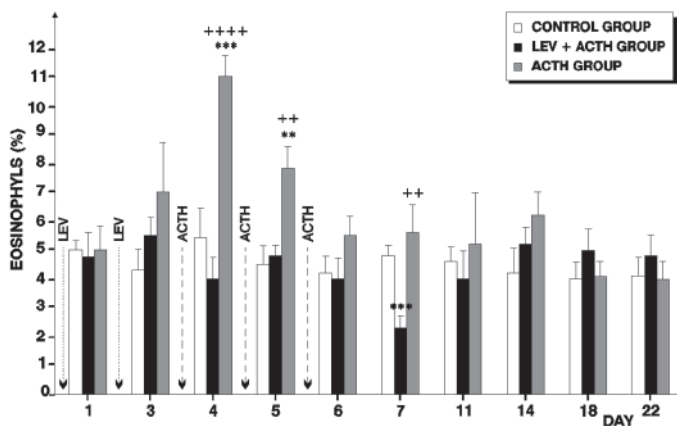


Fig. 7. Eosinophils (mean \pm SEM; %) in blood of boars treated with ACTH (10 μ g/kg/ body mass/day intravenously for 3 days; ACTH group, n = 6), levamisole (2.5 mg/kg/ body mass/day intramuscularly for 3 days) plus ACTH at above dosage (LEV+ACTH group, n = 6), and saline (1 mL intramuscularly for 6 days; control group, n = 6). Significant differences between groups: LEV+ACTH vs. control and ACTH vs. control (** P <0.01; *** P <0.001); LEV+ACTH vs. ACTH (++) P <0.01; +++ P <0.0001).

Discussion

Previous studies have shown that different types of stressor and intensity of stress conditions determine the process of changes in the hypothalamic-pituitary adrenocortical axis and immune response during stress. Also, the duration of stress, i.e. acute and chronic stress situations, differently influence the normal defence reactions and homeostasis (MUNCK et al., 1984). The objectives of the study were to justify the applicability of levamisole in a recommended immunomodulatory dose of 2.5 mg/kg body mass over three days before stress induction by three-day ACTH application in young boars.

The cortisol concentration is not always consistently changed by an acute stressor such as elevation of temperature, even if other signs of stress, especially behavioral signs, are clear (HICKS et al., 1998). Also, previous reports have shown that a challenge with ACTH or mild stressor conditions may produce differences among pigs in adrenocortical responsiveness (BROWN-BORG et al., 1993; HICKS et al., 1998). In the present study, the control levels of cortisol were comparable to those generally obtained in pigs sampled by venipuncture (WALLGREN et al., 1994; BILANDŽIĆ et al., 2006). Both groups of boars treated with ACTH (ACTH and LEV+ACTH) showed a significant increase in serum cortisol concentration after ACTH injection on all three days of treatment. Literature reports describe a similar increase in cortisol concentration after a single dose of ACTH was administered to female pigs (HAUSSMANN et al., 2000; MWANZA et al., 2000). However, in our study, performed for three consecutive days, a significant increase in cortisol concentration was recorded on the day after the last ACTH dose in both ACTH treated groups of boars, as compared with control animals.

The effect of levamisole on differential leukocyte count in different stress conditions is not completely understood. The effect of levamisole on immune responses in pigs and cattle was found to be highly dependent on the animal's condition, the dosage used and the time of administration (SYMOENS and ROSENTHAL, 1977; BRUNNER and MUSCOPLAT, 1980; BLECHA, 1988). Due to these factors, the use of levamisole as an immunomodulating agent in stressed animals is limited (BLECHA, 1988). In the present study, both stressed groups of pigs showed a significant increase in total leukocyte count during ACTH administration. This is consistent with our previous studies (BILANDŽIĆ et al., 2006). However, the use of levamisole before stress treatment led to a significant increase in total leukocyte count in the period after ACTH treatment as compared with either the control group or the group of stressed animals that were not given levamisole. ACTH treatment induced a significant decrease in lymphocyte proportion during administration, while lymphopenia seemed to terminate upon discontinuation of ACTH treatment. The administration of levamisole prior to stress induction produced an increase in lymphocyte count in pigs. Especially on the first day of ACTH administration this group had a significantly higher percentage of lymphocytes in comparison with the ACTH group. In

fact, the group of boars treated with levamisole before ACTH application showed a 7.5% to 13.5% lymphocyte count increase during the period of ACTH injection in comparison with the group of boars that did not receive levamisole. In artificially reared pigs use of levamisole in the same dose (2.5 mg/kg body mass) normalized the diminished cellular immune response by enhancing immune response up to the values of control animals and also enhanced lymphocyte proliferation (HENNESSY et al., 1987).

In this study, there was a significantly elevated percentage of neutrophils in both groups of boars during stress induction. The percentages of neutrophilic cells decreased in the ACTH group of boars in comparison with control animals in the week after ACTH treatment. In different stressful situations in pigs, such as shipping (STULL and McDONOUGH, 1994; HICKS et al., 1998) or mixing (PUPPE et al., 1997), it has been shown that lymphocyte count decreases simultaneously with neutrophil increase. In this report, the use of levamisole before ACTH application decreased the neutrophils count by about 3.5% to 7.7% as compared with the ACTH group of pigs. Furthermore, the administration of levamisole led to faster normalization of neutrophils in the week after stress cessation, on days 11 and 14, as compared with ACTH treated pigs. Also, levamisole prevented eosinophil leukocyte cell increase during ACTH treatment, as opposed to ACTH treated pigs who showed a significantly increased eosinophil count. However, on the day after discontinuation of ACTH treatment, levamisole led to a decrease in the eosinophils in stressed pigs. In previous reports, levamisole orally administrated at six different dosages in cattle failed to normalize the dexamethasone-induced suppression of neutrophil function or lymphocyte response (ROTH and KAEBERLE, 1984). It has been suggested that levamisole may change balance between the cAMP and cGMP cyclic nucleotides by increasing intracellular levels of cGMP in leukocytes (AMERY, 1978; MULCAHY and QUINN, 1986). The authors suggest that modulation of a number of lymphocyte and macrophage functions through the mediation of cGMP may be responsible for the *in vivo* action of levamisole as an immunomodulation agent.

In our study, the use of levamisole and ACTH treatment or the combination of these treatments had no effect on blood erythrocyte concentrations and hemoglobin levels in treated animals. Acute stress situations, such as changes in temperature conditions or transportation, had no effect on hemoglobin concentrations in pigs (HICKS et al., 1998). However, treadmill exercise induced an increase in hemoglobin concentrations in pigs (ZHANG et al., 1992). It is known that this increase may be due to a decrease in plasma volume by a temporary shift of water out of the vascular into the interstitial space (VAN BEAUMONT et al., 1973), or the release of erythrocytes stored in the spleen to increase oxygen carrying (LONGHURST et al., 1986).

Studies have shown that the use of levamisole as an immunomodulator in stressed livestock is limited, although in some cases it produced positive immunomodulating

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effects in pigs and cattle (BLECHA, 1988). Consequently, the present study suggested that levamisole may influence immune responsiveness and normalize or restore suppressed immune responses during and after artificial stress caused by ACTH injection in pigs.

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Received: 6 June 2009

Accepted: 29 January 2010

BILANDŽIĆ, N., B. ŠIMIĆ, S. TERZIĆ, M. ŽURIĆ: Utjecaj levamizola na koncentraciju kortizola i promjene u perifernoj krvi svinja izloženih stresu. *Vet. arhiv* 80, 477-489, 2010.

SAŽETAK

Istraživanje utjecaja levamizola na promjene u perifernoj krvi i koncentraciju kortizola provedeno je u svinja u stresu izazvanom davanjem adrenokortikotropnog hormona (ACTH). Pokus je proveden na nerastima švedskoga landrasa, u dobi 6 do 7 mjeseci, podijeljenih u tri skupine. Svinje u prvoj skupini dobivale su levamizol (2,5 mg/kg/tj. mase/dan, i.m.) tijekom tri dana i ACTH (10 µg/kg/tj. mase/dan, i.v.) tijekom 3 dana, a u drugoj skupini ACTH (10 µg/kg/tj. mase/dan, i.v.) također tijekom slijedeća 3 dana. Treća skupina bila je kontrola koja je dobivala fiziološku otopinu i.m. 6 dana. U pokusu je praćen broj leukocita, diferencijalna krvna slika, broj eritrocita, koncentracije hemoglobina i kortizola. Tijekom trodnevnog davanja ACTH kao i dan nakon zadnje primjene u obje pokusne skupine zabilježeno je povećanje kortizola u serumu. Istovremeno je primjena ACTH izazvala značajno povećanje ukupnog broja leukocita, ali i smanjenje broja limfocita. Primjenom levamizola prije izazivanja stresa izazvalo se povećanje ukupnoga broja leukocita 16 dana nakon prestanka davanja ACTH. U obje pokusne skupine svinja utvrđeno je povećanje broja neutrofila drugi i treći dan primjene ACTH. Međutim, davanje levamizola prije izazivanja stresa dovelo je do brže uspostave fiziološkoga udjela neutrofila. U svinja koje su dobile i levamizol i ACTH nije došlo do porasta udjela eozinofila kao što je to zabilježeno u skupini koja je dobila samo ACTH. S obzirom na dobivene rezultate istraživanja može se pretpostaviti da primjena levamizola može posredno utjecati na imunوسي odgovor u svinja tijekom i nakon stresa potaknutog s ACTH.

Ključne riječi: levamizol, adrenokortikotropni hormon, stres, imunomodulacija, nerasti, leukociti
