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The influences of immune modulation with levamisole and polyoxyethylene-polyoxypropylene copolymers on the immunohematological, serum biochemical parameters and intestinal histocytomorphology of weaned pigs

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ABSTRACT

The objectives of this study were to investigate whether or not the synthetic compounds, levamisole (LEVA) and polyoxyethylene-polyoxypropylene (POE-POP) copolymers, well-known to act as immunomodulators (IMs) in swine, may positively influence the cellular and humoral immune parameters of weaned pigs, without negatively affecting their hematological (HE), serum biochemistry (SB) and gut histocytological (HC) homeostasis. The pigs from a commercial swine farm were weaned at 26 days of age, randomly divided into

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3 groups comprising 20 animals each, and kept in separate pens within the same rearing facility. At the age of 28 days (Day 0 of the experiment), the pigs were treated as follows: (1) control pigs perorally (p.o.) received 10 mL of saline, and the principals were p.o. treated with a single dose of 10 mL of either: (2) LEVA with 2.5 mg/kg body weight of the anthelmintic drug or (3) POE-POP with 2.5 mg/mL of the copolymer preparation. The experiment was conducted over 35 days, and 7 pigs per group were sampled for peripheral blood at 7 day intervals, starting at Day 0 before the treatments for immunohematology and SB analyses. At either Day 0 or Day 35 of the experiment 5 pigs per group were euthanatized and sampled for gut HC. The POE-POP-treated pigs had increased proportions of lymphocytes ($P<0.05$) at Day 35. These pigs had higher levels of total serum immunoglobulins ($P<0.05$) at Day 14. The LEVA-treated pigs had decreased proportions of lymphocytes ($P<0.05$) at Day 14, although their total leukocyte count was similar to that recorded in the controls. None of the tested IMs affected the values of HE parameters, indicating that they did not cause any harmful side effects during the observation period of 5 weeks following the treatments. Minor oscillations in SB parameters were observed, but their values were within the normal range for swine and in accordance with the ages of the pigs. The HC features of gut mucosa in the pigs from the principal groups showed very mild damage and were quite normal for farm pigs exposed to natural infections, indicating that the tested IMs did not induce any additional HC changes. The obtained results imply that immunomodulating treatment with tested IMs was not associated with any adverse effects on the monitored HE, SB and gut HC parameters, and thus, does not suggest any putative impairment in the general health status of weaned pigs throughout the experimental period.

Key words: immunemodulation, levamisole, copolymers polyoxyethylene-polyoxypropylene, hematology, serum biochemistry, gut histocytology, homeostasis, pigs

Introduction

Over the past few decades the scientific efforts of veterinary research have been dedicated to the understanding of animal infectious diseases, including the genomics and proteomics of pathogenic microbiota, host innate and adaptive immunity, and therapeutic strategies. In contrast, very little is known about the prevention of diseases through dietary and non-dietary approaches because these problems have been solved with the addition of antibiotic growth promoters (AGP). AGP have been widely used in the pig industry, particularly during the weaning transition when pigs are subjected to major stressful events, making them highly susceptible to gut disorders (LALLES et al., 2007). Since 2006 the EU has put a ban on such practices in the production of food animals. A considerable amount of research has been focused on the development of alternatives to AGP to maintain swine health and performance, resulting in many excellent reviews and research papers on natural and synthetic immunomodulators (GALLOIS and OSWALD, 2008; GALLOIS et al., 2009; VALPOTIC et al., 2013; VALPOTIĆ et al., 2014) and bioactive substances (LALLES et al., 2009; THACKER, 2013). However, due to the lack of data on the bioactive components of particular substances tested as a potential alternative to AGP, it has been difficult to prepare and standardize the agents of equivalent potency. Thus, the literature on their influence on porcine immunity and growth remains inconclusive as yet (GALLOIS et al., 2009). Namely, such alternatives should impact positively the health and performance of pigs, without inducing any adverse effects in the animals and their products, in order to be fully acceptable for farmers, feed manufacturers and consumers, and to be environmental friendly. Concomitantly, pharmacokinetic studies are necessary

to understand the precise biological effects of these substances which have putative immunomodulating properties, recently termed immunobiotics (IBCs), and to clarify the relationships between the immunomodulations induced and the general health status of pigs (ŠPERANDA and VALPOTIĆ, 2012). An enormous number of empirical studies of the exogenous effects of manipulation of the immune system of pigs and other food animals by IBCs of natural origin and feed additives have been carried out (VALPOTIĆ, 2000; BLECHA, 2001; GALLOIS et al., 2009; BAILEY, 2009).

Among synthetic IBCs, promising results have been obtained with levamisole (LEVA) and polyoxyethylene-polyoxypropylene (POE-POP). LEVA is chemically defined as a levo isomer of 2,3,5,6 tetrahydro-6-phenylimidazole (2,1-b) thiazole (Nilverm®, PLIVA, Zagreb, Croatia), well known as an anthelmintic drug with potent immunostimulating and adjuvanticity activities (BOŽIĆ, 2000; BOŽIĆ et al., 2002; BOŽIĆ et al., 2006; BILANDŽIĆ et al., 2010). POE-POP is a nonionic block copolymer under the patented BASF name reverse tetronic polyol T150R1 (USA patent no. 5,234.683/93) also known as Polyphore 32:5 (CytRx, Atlanta, GA, USA) with adjuvant activity capable of enhancing both humoral and cellular immunity (HUNTER and RAGLAND, 1993; ŠVER et al., 1996; VIJTIUK et al., 2005; VALPOTIĆ et al., 2013).

The literature dealing with LEVA and POE-POP as immunomodulators/adjuvants and potential alternatives to AGP, and their impact on pig immunity and performance is rather extensive. Since the late 1970s, LEVA has been proposed as a potent immunostimulatory agent for restoration of depressed humoral and cellular immune responses due to stressful events and infections accompanying the intensive production of farm animals (BLECHA, 2001), including pigs (HENNESSY et al., 1987; VALPOTIĆ et al., 2009a). POE-POP has been already proposed and patented as an immunomodulatory agent for prophylaxis and therapy of infections, as well as a potent adjuvant in farm animals, including pigs (HUNTER and RAGLAND, 1993; VALPOTIĆ et al., 2013). When given solely by oral route to weaned pigs, the agent acted as a powerful growth promoter (ŠPERANDA et al., 2009) and significantly increased the proportion of peripheral blood lymphocytes (PBL) (VALPOTIĆ et al., 2013).

However, data are still scarce on the potential adverse effects of perorally applied synthetic IBCs, such as LEVA and POE-POP, on porcine health status, particularly in terms of changing their hematological (BOŽIĆ and MRLJAK, 2001; ŠPERANDA et al., 2009) and biochemical parameters (BILANDŽIĆ et al., 2010), and inducing alterations of histocytology within the GALT, as their initially expected effects in this compartment (VIJTIUK et al., 2005).

The current study is a continuation of our previous research dealing with the efficacy of perorally applied LEVA and POE-POP as immunomodulators in recruitment of systemic and intestinal immune cell subsets of weaned pigs (VALPOTIĆ et al., 2013; VALPOTIĆ et al., 2014), and it was undertaken to investigate if these synthetic compounds may also positively influence peripheral blood cellular and molecular immune and non-immune

parameters of weaners, without negatively affecting their hematological, biochemical and gut histocytological homeostasis.

Materials and methods

Pigs. Sixty crossbred pigs were used (Topigs®), both females and castrates, with a body weight of approximately 6.8 kg, the progeny of six litters (from 3rd parity sows) from a commercial swine farm in eastern Croatia. The pigs were weaned at 26 days of age, housed, managed and fed with a standard weaner diet (without antimicrobials or growth promoters) according to the rearing technology of the farm. Experimental and animal management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC).

Experimental design and treatment. The weaned pigs were randomly divided into three groups comprising 20 animals each, ear-tagged with numbers 1- 20 and kept in the same rearing facilities of the commercial farm in separate pens as described earlier (VALPOTIĆ et al., 2014). After two days of accommodation, at 28 days of age (Day 0 of the experiment), the pigs were treated as follows: (1) control pigs perorally (p.o.) received 10 mL of saline, and the principals were p.o. treated with a single dose of 10 mL of either (2) LEVA (Nilverm®, Pliva, Zagreb, Croatia) with 2.5 mg/kg body weight (BW) of the anthelmintic drug or (3) POE-POP (CytRx, Atlanta, GA, USA) with 2.5 mg/mL of the copolymer preparation, as detailed earlier (VALPOTIĆ et al., 2013, VALPOTIĆ et al., 2014). The experiment was conducted over a period of 35 days, and 7 pigs per group were sampled for peripheral blood at seven day intervals starting at Day 0 before the treatment. At Day 0 (prior to the treatments) and Day 35 of the experiment, 5 pigs per group were euthanatized by intracardial injection of 0.3 mL/kg of T61 preparation (Hoechst®, München, Germany) and sampled for histopathology.

Sampling. Blood samples (10 mL) were collected from the v. cava cranialis using vacutainers (Beckton Dickinson, Plymouth, UK) and separated into two aliquots, one (2 mL) in plastic tubes with disodium EDTA (Sigma, St. Louis, USA) as an anticoagulant (1 mg/mL^{-1}) for immunohematology, and one (8 mL) into glass tubes for serum biochemistry. Immediately following euthanasia at both Day 0 and Day 35 of the trial, specimens (1 cm) of the mid jejunum and ileum and part (0.5 cm) of the mesenteric lymph node (MLN) were taken from each of 5 pigs per group, and fixed in 10% paraformaldehyde - phosphate buffered saline (PBS) solution (pH 7.2) for 24 hours, until used for histopathology analysis.

Immunhematological analyses. Total leukocyte count was determined using an automated counter (System 9120, Serono Baker, Pennsylvania, USA). The blood smears were prepared and stained according to the May Grűnwald-Giemsa technique, and examined by a microscope Olympus BX 41 under immersion magnification in order to determine the differential blood counts. The relative ratio of leukocyte subpopulations

(lymphocytes, neutrophils and eosinophils) was acquired in relation to their total counts. The approximate value of total serum immunoglobulins (Igs) due to the presence of alpha globulins, was obtained after determining the values of total proteins and albumin, with original reagent kits from Olympus Diagnostica, using an automated biochemistry analyzer (Olympus AU 600, Olympus Diagnostic, Hamburg, Germany) and by subtracting the value for albumin from that obtained for total serum proteins. The numbers of erythrocytes and thrombocytes, as well as the levels of hemoglobin and hematocrits, were determined by the standard methods using an automated counter (Serono Baker System 9120, Pennsylvania, USA).

Serum biochemistry analyses. The serum profiles of the hepatic enzymes: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and creatinine kinase (CK) and metabolites (glucose, creatinine, urea, total cholesterol, total triglycerides, total proteins and albumin) were determined according to the standard methods, using original reagent kits from Olympus Diagnostica (OSR6204 for ALP, OSR6509 for AST, OSR6507 for ALT, OSR6520 for GGT, OSR6279 for CK, OSR6521 for glucose, OSR6578 for creatinine, OSR6534 for urea, OSR6516 for total cholesterol, OSR61118 for total triglycerides, OSR6532 for total proteins and OSR6502 for albumin) by an automated biochemistry analyzer (Olympus AU 600, Olympus Diagnostic, Hamburg, Germany).

Histopathological examination. After fixation in a 10% paraformaldehyde - PBS solution the specimens of jejunum, ileum and MLN were processed for histopathological examination by dehydration in 70% and 96% ethanol for 1 hour, and by two immersions in 100% ethanol for 1 hour. The specimens were incubated overnight in chloroform at 56 °C and then transferred into a mixture of chloroform and Paraplast (1:1) at 56 °C for 1 hour. Further, they were immersed twice in Paraplast (Paraplast I and Paraplast II) embedding medium (Sigma, Deisenhofen, Germany) for 1 hour at 56 °C. After cooling at room temperature, the Paraplast-embedded specimens were cut by microtome (Reichert-Jung, Germany) into 5-6 μm thick serial sections and floated on a water bath heated to approximately 50 °C. The selected sections were picked up with the 2% APES (3-aminopropyl-triethoxysilane, Sigma, St. Louis, USA) in acetone precoated slides and dried horizontally on a warming tray overnight at 37 °C. The sections were dewaxed in xylene (twice or 10 minutes), hydrated in graded alcohol solutions (for 5 minutes n 100%, 96%, 80% and 70% ethanol) and immersed in distilled water**.** After rinsing in distilled water (twice for 5 minutes), the sections were immersed in hemalaun (for 10 minutes), rinsed in tap water and immersed in eosin (for 2 minutes). Then the sections stained with hemalaun-eosin (HE) were again rinsed in distilled water (twice for 5 minutes) and dehydrated in graded alcohol solutions (70%, 80%, 96% and 100% ethanol), enlightened by short immersion in xylene and embedded in Canada balsam. The sections of the specimens sampled were dried and examined for histopathological changes under a light

microscope with a built-in camera (Nikon Microphot -FXA, Japan). The selected areas of each tissue specimen section were photographed.

Statistical analysis. Numerical data were analyzed by the Student's *t* test for dependent samples using the Statistica SixSigma software (StatSoft, Inc.). The significance of differences between the values obtained for the pigs in the control group and those obtained in the pigs treated with either of the immunomodulators applied were considered as significant at $P<0.05$ and lower values.

Results

Immunological findings. The pigs that received POE-POP had higher proportions of lymphocytes ($P<0.05$) at Day 35, although their total leukocyte number was not significantly increased. These pigs had also higher levels of total serum Igs ($P<0.05$) at Day 14 of the experiment (Table 1).

Table 1. Cellular and molecular immune parameters in the peripheral blood of weaned pigs treated with immunomodulators LEVA and POE-POP

Item	0	7	14	21	28	35	SEM*
Leukocytes ($\times 10^9$/L)							
Control	21.54	21.94	24.07	25.08	25.89	25.21	2.61
LEVA	19.31	19.57	24.87	22.90	24.17	32.07	2.34
POE-POP	19.60	21.20	23.70	26.82	25.17	30.97	2.73
Lymphocytes (%)							
Control	48.61	50.45	58.29	56.37	59.78	59.43	1.92
LEVA	50.14	52.86	46.43[a]	65.00	54.57	56.43	4.08
POE-POP	53.29	55.00	60.14	64.56	66.14	8.71[a]	3.60
Neutrophils (%)							
Control	51.43	45.87	43.57	41.83	37.42	38.51	4.29
LEVA	52.29	44.00	50.00	33.14	44.29	41.29	4.12
POE-POP	45.29	45.14	40.14	34.67	33.43	33.83	5.17
Eosinophils (%)							
Control	3.14	4.25	3.00	2.00	5.00	5.00	0.63
LEVA	4.40	3.33	4.33	2.67	1.67	2.25	0.84
POE-POP	4.40	2.00	2.00	3.00	2.50	2.00	0.69
Total Igs (g/L)							
Control	25.35	27.08	26.24	28.98	28.90	29.42	2.15
LEVA	27.70	28.73	26.69	29.89	27.70	31.97	1.58
POE-POP	27.77	32.30	32.68[a]	29.53	32.63	33.50	1.92

Data are presented as mean ± SEM (means of a single pen comprised seven pigs each). Means that differ significantly from the control group ($P<0.05$) are marked with a superscript [a]. * - Pooled SEM.

Table 2. Hematological parameters of weaned pigs treated with immunomodulators LEVA and POE-POP

Item	0	7	14	21	28	35	SEM*
Erythrocytes ($\times 10^{12}$/L)							
Control	5.33	5.88	5.33	5.16	6.13	5.32	0.31
LEVA	5.34	5.97	5.77	5.09	5.56	6.09	0.25
POE-POP	5.39	6.08	5.73	4.86	5.25	5.61	0.28
Hemoglobin (g/L)							
Control	94.71	101.3	93.57	95.17	111.6	99.57	6.64
LEVA	94.00	102.6	93.86	92.86	101.4	108.1	3.56
POE-POP	92.00	101.7	98.14	88.71	94.43	100.4	3.67
Hematocrit (L/L)							
Control	0.30	0.32	0.28	0.29	0.34	0.30	0.35
LEVA	0.29	0.33	0.30	0.27	0.30	0.34	0.03
POE-POP	0.28	0.32	0.31	0.26	0.29	0.31	0.04
MCV (f/L)							
Control	55.57	53.63	52.26	55.03	55.60	56.03	1.69
LEVA	54.61	54.01	52.24	53.83	54.24	55.11	1.13
POE-POP	54.43	52.66	53.77	53.33	54.67	55.19	1.04
MCHC (g/L)							
Control	316.4	321.7	336.6	335.3	328.7	336.3	6.01
LEVA	323.3	318.6	312.0	339.4	337.0	322.9	4.32
POE-POP	314.1	317.9	319.6	344.9	330.3	324.9	4.47

Data are presented as mean ± SEM (means of a single pen comprised seven pigs each). * - Pooled SEM.

However, the pigs treated with LEVA had significantly decreased proportions of lymphocytes ($P<0.05$) at Day 14, although their total leukocyte count was similar to that recorded in the saline-treated controls at Day 14 of the experiment (Table 1). Interestingly, a trend of a fall in the proportions of neutrophils and eosinophils, either in all of the groups or just in the principals, was observed with advancing age. An age-dependent, although not significant, increase in the levels of total Igs was observed in the pigs from all three groups, reaching peak values at the end of the experiment.

Hematological findings. We assessed the general health status of the experimental pigs by hematological analyses on a weekly basis. None of the tested immunomodulators affected the values of hemogram and erythrocyte constants, indicating that they did not cause any harmful side effects during the observation period of 35 days following the treatments (Table 2).

Table 3. Serum levels of hepatic enzymes in weaned pigs treated with immunomodulators LEVA and POE-POP

Item	0	7	14	21	28	35	SEM*
ALP (U/L)							
Control	325.1	256.6	222.3	231.1	239.0	284.1	41.7
LEVA	268.8	192.1	278.0	330.6	306.4	291.5	25.1
POE-POP	323.1	280.0	199.4	191.3	232.4	254.6	25.2
AST (U/L)							
Control	32.7	51.6	47.3	50.6	42.1	48.4	7.1
LEVA	27.0	41.3	36.7	41.9	51.1	63.8	6.4
POE-POP	33.7	31.2	47.0	33.8	49.6	39.0	5.8
GGT (U/L)							
Control	48.0	42.1	37.6	31.6	39.6	31.4	5.9
LEVA	32.7	27.7	30.3	29.6	27.3	29.5	4.4
POE-POP	38.4	32.8	24.0	20.7	25.7	22.1	4.0
ALT (U/L)							
Control	42.0	33.1	35.3	37.9	42.1	51.9	3.9
LEVA	33.7	23.7	31.0	36.4	39.7	47.0	3.0
POE-POP	36.9	28.0	28.4	24.2	36.6	44.3	3.2
CK (U/L)							
Control	238.6	1598	736.7	970.0	2032	2075	689
LEVA	251	1105	664	501.1	556.3	867.2	196
POE-POP	269.7	291.2	945.9	428	689.9	653.0[a]	197

Data are presented as mean ± SEM (means of a single pen comprised seven pigs each). Means that differ significantly from the control group ($P<0.05$) are marked with a superscript [a]. * - Pooled SEM.

Serum levels of hepatic enzymes and metabolites. Serum biochemical parameters, such as liver enzymes (Table 3) and metabolites (Table 4), were also analyzed on a weekly basis, in order to monitor the general health status of the pigs during treatment with the immunomodulators tested. While the levels of ALP, AST, GGT and ALT remained unaffected by both immunomodulators applied during the experiment, the concentration of CK was decreased in the pigs treated with POE-POP ($P<0.05$) at Day 35 of the experiment (Table 3).

The pigs treated with LEVA had increased levels of creatinine ($P<0.05$) at Day 35 and glucose ($P<0.05$) at Days 14 and 28, and decreased concentration of total proteins ($P<0.01$) at Day 28 of the experiment (Table 4).

Conversely, the POE-POP-treated pigs had higher levels of triglycerides ($P<0.05$) at Day 14 of the experiment (Table 4).

The pigs treated with LEVA had increased levels of creatinine ($P<0.05$) at Day 35 and glucose ($P<0.05$) at Days 14 and 28, and decreased concentration of total proteins ($P<0.01$) at Day 28 of the experiment (Table 4).

Table 4. Serum levels of metabolites in weaned pigs treated with immunomodulators LEVA and POE-POP

Item	0	7	14	21	28	35	SEM*
Urea (mmol/L)							
Control	2.87	4.73	4.79	4.41	4.35	3.43	0.35
LEVA	2.65	5.33	4.50	4.07	3.41	4.40	0.40
POE-POP	3.19	4.66	5.40	5.09	4.49	3.92	0.30
Cholesterol (mmol/L)							
Control	1.97	1.92	1.83	2.52	2.62	2.68	0.16
LEVA	1.99	1.77	1.86	2.45	2.89	2.70	0.14
POE-POP	2.51	1.88	1.88	2.58	2.47	2.63	0.19
Creatinine (µmol/L)							
Control	124.1	111.9	111.4	95.86	98.14	83.00	4.88
LEVA	121.5	101.7	102.3	87.83	92.17	101.7[a]	4.43
POE-POP	123.3	103.8	97.42	89.67	94.71	89.86	3.93
Tryglicerides (mmol/L)							
Control	0.53	0.65	0.51	0.63	0.74	0.54	0.08
LEVA	0.46	0.61	0.54	1.05	0.73	0.70	0.09
POE-POP	0.50	0.52	1.05[a]	0.72	0.70	0.67	0.12
Glucose (mmol/L)							
Control	6.19	5.34	5.29	5.93	4.90	6.48	0.39
LEVA	6.62	4.85	6.72[a]	6.11	6.77[a]	6.55	0.24
POE-POP	5.93	6.60	5.09	4.87	5.47	5.44	0.32
Total protein (g/L)							
Control	58.71	54.71	55.86	55.71	63.14	56.57	2.01
LEVA	59.00	53.85	53.86	50.43	52.43[b]	52.67	1.36
POE-POP	57.86	53.00	55.71	56.33	57.29	56.00	2.07
Albumin (g/L)							
Control	33.36	27.63	26.32	29.47	29.63	27.15	1.57
LEVA	31.30	25.13	23.97	23.74	24.72	24.85	1.01
POE-POP	28.95	25.24	24.54	23.66	24.65	23.70	1.36

Data are presented as mean ± SEM (means of a single pen comprised seven pigs each). Means that differ significantly from the control group are marked with a superscript [a] ($P<0.05$) and [b] ($P<0.01$). * - Pooled SEM.

Conversely, the POE-POP-treated pigs had higher levels of triglycerides ($P<0.05$) at Day 14 of the experiment (Table 4).

Small intestinal histocytological findings. Apical necrosis of the villi and desquamation of the epithelium were visible in the mid jejunum of the pigs from the saline-treated control group (Fig. 1a). In the lamina propria (LP) moderate histiocytosis and infiltration of lymphocytes as well as of globular leukocytes (GL) at the basis of the Lieberkühn crypts were observed. Solitary lymphoid follicles could be noticed within the submucosae.

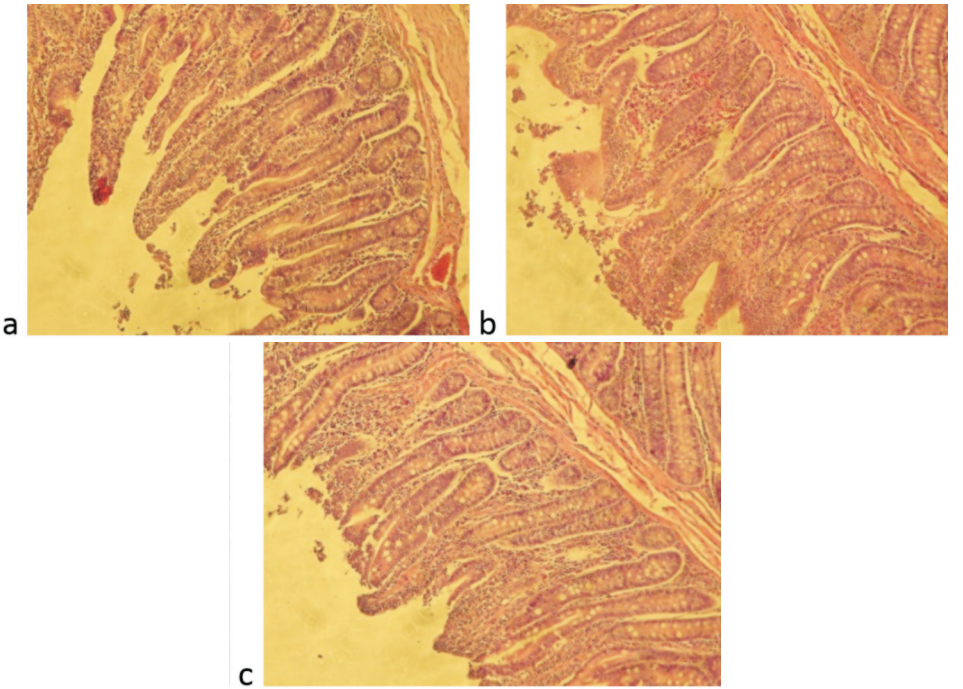

Fig. 1. Mid jejunum of 63-day-old pigs from the saline-treated control group (a) and from the principal groups treated p.o. with either LEVA (b) or POE-POP (c) after 35 days following the treatments. H&E, ×10.

A mild focal desquamation of the epithelium and moderate infiltration of mononuclear leukocytes (MNL) with a multitude of GL in the LP of the mid jejunum were observed in the LEVA-treated pigs (Fig. 1b). In the POE-POP-treated pigs, similar findings were noticed, but the epithelial desquamation was even less expressed (Fig. 1c). In the mid ileum of the control pigs, the villi became thicker and tended to amalgamate in some places (Fig. 2a). Focal accumulations of GL and moderate infiltration of MNL could be seen in the LP. The prominent hyperplasia of the PP and edema of the submucosae were also visible.

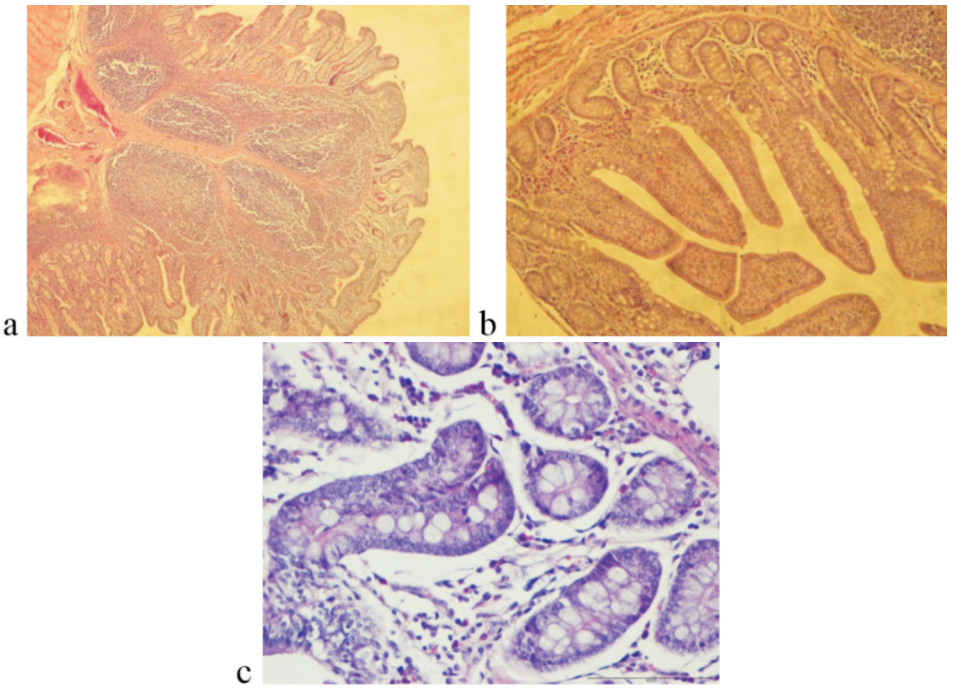

Fig. 2. Mid ileum of 9-week-old pigs from the saline-treated control group (a) and from the principal groups treated p.o. with either LEVA (b) or (POE-POP) at Day 0 (or 4-weeks of age) after 5 weeks following the treatments. H&E, ×4, ×10 or ×20, respectively.

The pigs treated with LEVA had moderate edema of the submucosae, less intensive infiltration of MNL and less numerous GL in the LP, as well as normally developed PP (Fig. 2b). The POE-POP-treated pigs had milder epithelial damage with less prominent edema of the mucosa and moderate infiltration of MNL and GL within the ileal LP (Fig. 2c).

Considerably expressed primary and secondary lymphoid follicles within the MNL were visible in the control pigs, along with sinus histiocytosis as well as dense micro follicular accumulation of lymphocytes (Fig. 3a).

Strong follicular hyperplasia in the medulla of the MLN from the LEVA-treated pigs and sinus histiocytosis, with more numerous GL and less numerous eosinophils, were observed (Fig. 3b). In the MLN from the POE-POP-treated pigs, a similar finding was recorded, with visible primary and secondary follicles and sinus histiocytosis of less numerous GL (Fig. 3c).

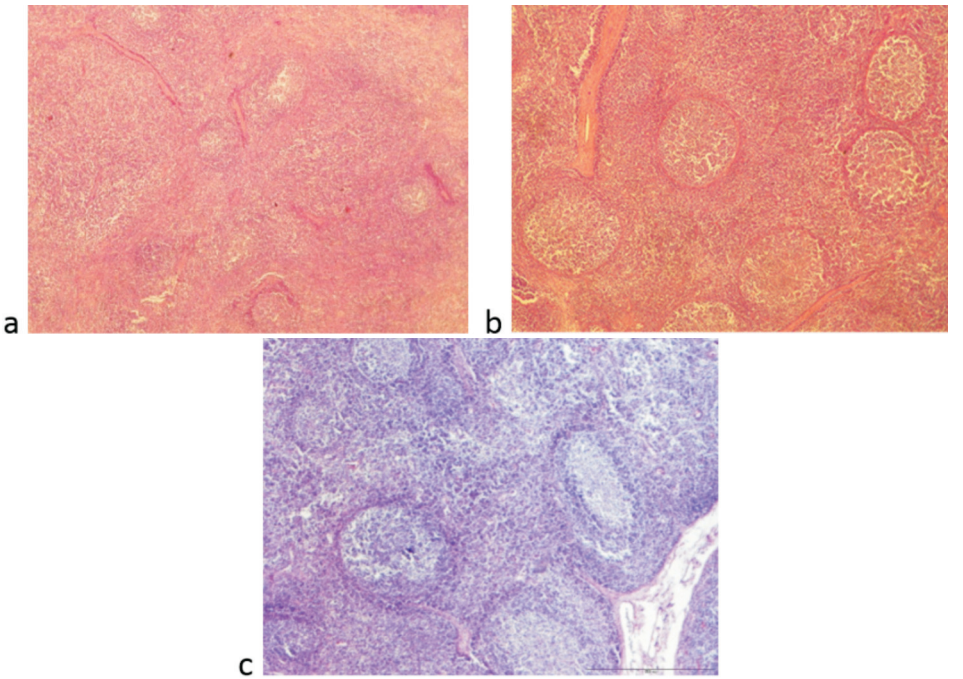

Fig. 3. Lymphoid follicules in the MLN of 9-week-old pigs from the saline-treated control group (a) and from the principal groups treated p.o. with either LEVA (b) or (POE-POP) at Day 0 (or 4-weeks of age) after 5 weeks following the treatments. H&E, ×4.

Discussion

Regarding the limited reports on the potential adverse effects of LEVA, and in particular of POE-POP, which are known to be potent adjuvants or IBCs in various animal species including swine, we considered that data should be presented on their safety for the health status of tested pigs in our recent studies (VALPOTIĆ et al., 2013; VALPOTIĆ et al., 2014). Since there is no evidence that these compounds are degraded during their passage through the gastrointestinal tract of treated animals, the risk for consumers is also an important aspect. In these studies we monitored the gut health status of tested pigs by daily clinical observation and scoring the incidence/severity of diearrhea, and by weekly bacteriological examination. In the current study we assessed the general health status of the IBCs-treated pigs by hematological and biochemical analyses on a weekly basis. None of the tested compounds affected the values of hemogram and erythrocyte constants or liver enzymes (with the exception of lower CK values in the POE-POP-treated pigs), or caused any serious changes in the serum profiles of metabolites, where changes were evident in either creatinine, glucose and total protein levels ($P<0.05$ and $P<0.01$)

or in triglyceride levels ($P<0.05$) in the LEVA- or POE-POP-treated pigs, respectively. Similarly, there were no major pathological changes in the histocytology of the jejunal/ileal mucosa and GALT tested. The results indicated that the use of LEVA and POE-POP as IBCs in pig nutrition (oral application) does not pose a risk for consumers. Our previous observation is in accordance with this that LEVA (when used as an adjuvant for F4ac$^+$ non-ETEC vaccine) did not compromise the non-immune defense mechanism of the intestinal mucosa (protected by a mucus layer) as the mucus-producing goblet cells of weaned pigs were not adversely affected, and it exhibited an anti-inflammatory effect as the frequency of ileal mast cells was not significantly changed (VALPOTIĆ et al., 2009b). The later finding could be supported by the observation that porcine basophilic granulocytes (precursor cells for mastocytes) were unaffected by treatment with LEVA (BILANDŽIĆ et al., 2010).

Nevertheless they exhibited efficacy in modulating immunohematological parameters by both significantly depressing (LEVA) and stimulating (POE-POP) PBL counts ($P<0.05$) at Day 14 and at Day 35 of the experiment, respectively. POE-POP also induced significant elevation of total Igs level ($P<0.05$) at Day 14 following the treatment. Such short-term changes could be ascribed to individual fluctuations due to the exposure of the pigs to stressful events accompanying weaning and immune challenges, as suggested for LEVA (KUMAR et al., 1999). Interestingly, BOŽIĆ and MRLJAK (2001) reported a significant decrease in leukocytes and a simultaneous increase in PBL numbers in pigs primed with *i. m.* applied LEVA prior to specific immunization with the vaccine candidate F4ac$^+$ non-ETEC strain, and challenge infection with the homologous ETEC strain. However, when the agent was given i.m. solely for three consecutive days (at the same dose as we did) to boars aged 210 days, total leukocyte counts and the proportion of neutrophil granulocytes were significantly increased, whereas PBL and monocyte percentages remained unaffected (BILANDŽIĆ et al., 2010). These authors also recorded significantly elevated serum IgG levels (predominant class of Igs within the globulin fraction), which is not in accordance with our finding that LEVA treatment had no effect on serum levels of total Igs. This could be ascribed to the fact that the pigs in our study were treated with a single dose of LEVA and by a different route (p.o.) at the age of 28 days, when they were not immunologically competent or even immunologicaly compromised due to stressful events accompanying weaning.

Literature dealing with the impact of POE-POP treatment on the porcine health parameters tested in our study is very rare. In fact, there is a study by ŠPERANDA et al. (2009) which contrasts with our data obtained for POE-POP as a potent IBC for peripheral blood molecular (total Igs) and cellular (PBL) immune parameters. These authors reported an increased trend of PBL counts, but it was not significant, which is not consistent with our finding at the end of the trial. In general, the obtained immunohematological parameters only differed slightly from the reference values for young pigs (THORN, 2000).

Our data obtained for the hemogram and erythrocyte constants of the POE-POP-treated pigs agree well with the results on hematological parameters obtained by

ŠPERANDA et al. (2009), with the exception of the slightly lower erythrocyte counts (6.01 ± 0.45 *vs.* 5.61 ± 0.18×10^{12}/L; ref. values: 5.3-8.0×10^{12}/L) or hemoglobin levels (108.43 ± 6.27 vs. 100.4 ± 3.02 g/L; ref. values: 90-140 g/L) at Day 35 of the experiment, and they were within the range of reference values for weaned pigs (FRIENDSHIP et al., 1984). However, these pigs had values for erythrocytes (4.86 ± 0.31×10^{12}/L) and hemoglobin (88.71 ± 4.10 g/L) slightly below the reference values at Day 21 of the experiment. The pigs treated with LEVA had values for MCHC (312.0 ± 3.59 g/L) slightly lower than the range of normal reference values (320-360 g/L) at Day 14 of the experiment.

Further, while the data for the proportions of either PBL (45-68%) or neutrophils (29-52%) were almost within the range of normal values for weaned pigs (40-68% and 28-43%, respectively), the obtained values for either leukocytes (17-34×10^{9}/L) or eosinophils (1-5%) were increased (13-21×10^{9}/L) or decreased (4-14%), respectively, in relation to the reference values (FRIENDSHIP et al., 1984; THORN, 2000). The values for total serum Igs were found to be slightly higher (24-34 g/L) than the reference values (22.4-24.6 g/L) according to KANEKO et al. (2008), probably due to the effects of the IBC tested (particularly POE-POP), but also could be related to the differences in breed, and genetic and paragenetic factors between the tested pigs. However, the data obtained by quantitative histomorphometric analyses demonstrated that both LEVA and POE-POP, applied as mucosal adjuvants, were able to synergize the recruitment of IgA^{+} plasma cells and the release of specific anti-F4ac sIgA antibodies within the small intestinal mucosa of weaned pigs immunized with the vaccine candidate $F4ac^{+}$ non-ETEC strain (JANJATOVIĆ et al., 2008; VIJTIUK et al., 2005), which contrasts with the observations of VAN WAUWE and JANSSEN (1991), who reported that LEVA had no effect on levels of Igs or antibody formation. However, the immunostimulating potential of POE-POP to increase the serum level of total Igs in weaned pigs, as recorded in this study, is in accordance with an early finding of its ability to selectively elicit the formation of antibodies of protective isotypes (ALLISON and BYARS, 1986).

Generally, the values of most of the biochemical parameters (including liver enzymes and metabolites) tested were within normal ranges for swine, and in accordance with the ages of the pigs. Among the investigated liver enzymes, only the concentration of CK (653.0 ± 150 U/L) was significantly decreased in the pigs treated with POE-POP ($P<0.05$) at Day 35 of the experiment, although this value was within the range of the reference values (81-1586 U/L). Similarly to the hematological parameters, minor physiological oscillations of serum metabolites were observed during the experiment, with the exception of slightly lower concentrations of urea (2.65 ± 0.62 mmol/L) than the reference values (2.90-8.89 mmol/L) at Day 0 prior to the treatment of pigs with LEVA. The pigs treated with LEVA had significantly increased levels of creatinine at Day 35 ($P<0.05$), and glucose at Days 14 and 28 ($P<0.05$), as well as decreased levels of total protein at Day 28 ($P<0.01$). POE-POP induced higher levels of triglycerides ($P<0.05$) at Day 14 of the experiment. However, all these deviations were within the normal range according to KANEKO et al. (2008).

As the small intestinal mucosa was found to be only mildly damaged in the pigs from the principal groups, we assume that the IBCs tested did not induce any adverse histcytological changes. On the contrary, these IBCs provided immune protection against intraluminal microbiota as was suggested for LEVA (BOŽIĆ et al., 2003a,b; VIJTIUK et al., 2005; BOŽIĆ et al., 2006; JANJATOVIĆ et al., 2008; VALPOTIĆ et al., 2014) as well as for POE-POP (VALPOTIĆ et al., 2013). Such protection of the jejunal and ileal mucosal surfaces of the IBCs-treated pigs was characterized by more extensive infiltration of lymphoid and myeloid cells as compared to that in the untreated control pigs. The increased cellularity of the MLN and ileal PP, as well as the infiltration of dispersed and aggregated lymphatic tissues was observed within the small intestinal mucosa by histiocytes and eosinophils. However, these mucosal damages were found to be less severe than those observed in pigs experimentally immunized with attenuated the F4ac$^+$ non-ETEC vaccine candidate strain (VIJTIUK et al., 1995), indicating that the IBCs tested did not additionally damage the jejunal and ileal mucosa in the treated pigs. Finally, it should be emphasized that histocytological features were also quite normal for farm pigs exposed to natural infections most commonly caused by pathogenic ETEC strains. Under "natural conditions" their weaning is gradual process which is not complete until 10 to 12 weeks of age. This is however not normal rearing practice and pigs are today weaned abruptly at 3 to 4 weeks (LALLES et al., 2007). As a consequence, early weaning affects the ontogeny of immune functions, particularly the immune responses to oral delivery of feed and microbial antigens, including IBCs which is orchestrated by a well-developed intestinal mucosal immune system (BAILEY, 2009). The kinetics of postnatal development of porcine gut immune system (ŽUBČIĆ et al., 2014) may therefore play a critical role in determining the outcome of the responses to harmless dietary antigens, commensal bacteria and IBCs and harmful pathogens or their products (STOKES et al., 2004).

It could be concluded that a single p.o. immunomodulating treatment with either LEVA or POE-POP at the doses used in our study was not associated with any adverse effects on the monitored hematological, serum biochemical and small intestinal histocytology parameters, and thus, there is no suggestion of putative impairment of the general health status of the weaned pigs throughout the experimental period due to these treatments. However, as the final aim of using such synthetic compounds in swine production is to maintain and improve the pigs' health, performance and welfare, further studies of LEVA and POE-POP are needed in order to define precisely their site of action in the organism and the relationships between the immunomodulatory effects they induce as alternatives to AGP, and the health status of pigs.

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SAŽETAK

Ciljevi ovoga rada bili su istražiti mogu li, ili ne, sintetički spojevi levamisol (LEVA) i polioksietilenski-polioksipropilenski (POE-POP) kopolimeri, dobro znani da djeluju kao imunomodulatori (IM) u svinje, pozitivno utjecati na stanične i humoralne imunosne pokazatelje odbijene prasadi a da negativno ne djeluju na njihovu hematološku (HE), serumsku biokemijsku (SB) i crijevnu histocitološku (HC) homeostazu. Na komercijalnoj svinjogojskoj farmi prasad je bila odbijena u dobi od 26 dana, nasumično podijeljena u tri skupine od po 20 životinja u svakoj, te držana u odvojenim odjeljcima u istoj uzgojnoj nastambi. U dobi od 28 dana (= 0. dan pokusa), prasad je tretirana kako slijedi: (1) kontrolna prasad je per os (p.o.) primila 10 mL fiziološke otopine, a pokusna je prasad bila p.o. tretirana jednokratnom dozom od 10 mL ili (2) LEVA s 2,5 mg/kg tjelesne težine antihelmintičkog lijeka, ili POE-POP sa 2,5 mg/mL kopolimerskog pripravka. Pokus je proveden tijekom 35 dana, a od 7 prasadi po skupini uzimani su uzorci periferne krvi za imunohematološke i SB pretrage u razmacima od 7 dana počevši od 0. dana prije tretmana. Po pet prasadi iz svake skupine bilo je eutanazirano 0. i 35. dana pokusa radi uzimanja uzoraka za crijevnu HC. Prasad koja je primila POE-POP imala je veći udjel limfocita ($P<0,05$) 35. dana. Ta je prasad imala višu razinu ukupnih serumskih imunoglobulina ($P<0,05$) 14. dana. Prasad tretirana s LEVA imala je smanjen udjel limfocita ($P<0,05$) 14. dana, premda je ukupan broj njihovih leukocita bio sličan onom zabilježenom u kontrolne prasadi. Niti jedan od testiranih IM nije utjecao na vrijednosti HE pokazatelje, upućujući na to da nisu uzrokovali nikakve štetne popratne učinke tijekom razdoblja praćenja pokusa od pet tjedana nakon tretmana. Utvrđene su manje oscilacije pokazatelja SB, ali su te vrijednosti bile u rasponu normalnih vrijednosti za svinju i primjerene dobi prasadi. HC nalazi u sluznici crijeva prasadi pokusnih skupina pokazivali su vrlo blaga oštećenja i bili su gotovo normalni za farmsku prasad koja je izložena prirodnim infekcijama te upućuju na to da testirani IM nisu izazivali nikakve dodatne HC promjene. Dobiveni rezultati podrazumijevaju da imunomodulacijski tretman s testiranim IM nije bio povezan s bilo kakvim nepovoljnim učincima na HE, SB i crijevne HC pokazatelje.

Ključne riječi: imunomodulacija, levamisol, kopolimeri POE-POP, hematologija, serum, biokemijski pokazatelji, histocitološki nalaz, homeostaza, prasad