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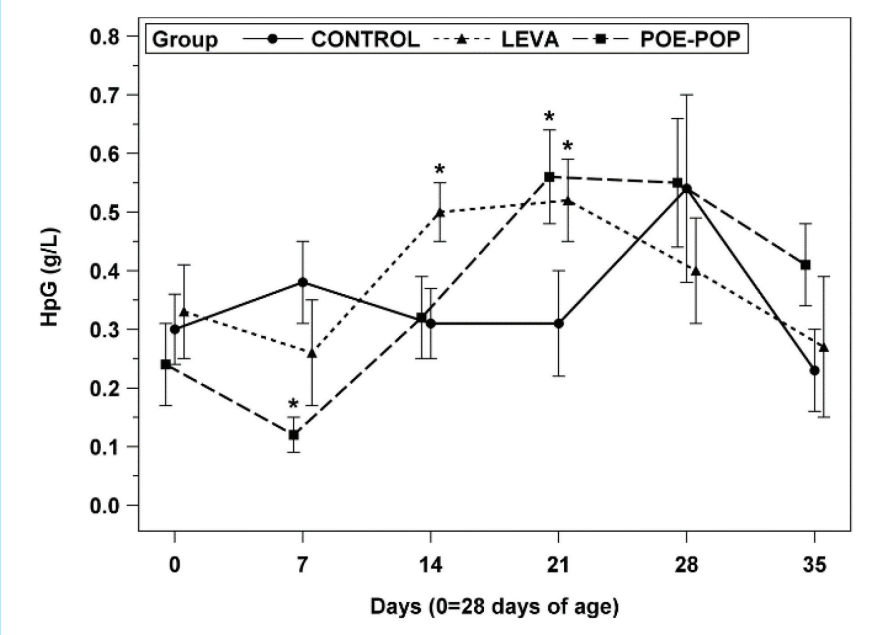

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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

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Original Research Article

DEVELOPMENT AND EVALUATION OF ANTIGEN CAPTURE ELISA FOR THE DETECTION OF INFLUENZA VIRUS A

Mahshid Aria[1], Masoud Reza Seifiabad Shapouri[2], Mohammad Roayaei Ardakani[1,3], Hossein Motamedi[1,3]*, Azadeh Yektaserasht[4], Amin Jaidari[5]

[1]Department of Biology, Faculty of Science, [2]Department of Pathobiology, Faculty of Veterinary Medicine, [3]Biotechnology and Biological Science Research Center, Shahid Chamran University of Ahvaz, Ahvaz, [4]Department of Pathobiology, Faculty of Veterinary Medicine, Shiraz University, Shiraz, [5]Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran

*Corresponding author, E-mail: motamedih@scu.ac.ir

Abstract: The aim of the present study was to develop an Antigen Capture ELISA (AC-ELISA) for the diagnosis of avian influenza virus infections. For this purpose, the nucleoprotein (NP) of the virus was captured by a monoclonal antibody (D'C4) and then detected using a rabbit polyclonal antibody. The developed AC-ELISA did not show cross-reaction with other viral and bacterial pathogens of poultry, while it was able to detect H9 serotype of avian influenza virus as well as H1 and H3 types of human influenza viruses. The sensitivity of this AC-ELISA for the detection of an H9 avian influenza virus strain H9N2 (A/Chicken/Iran/AH-1/06) was 10 times greater than a hemagglutination assay and was comparable with the sensitivity of the RT-PCR method. Furthermore, this method could recognize the influenza virus in tracheal swabs of experimentally infected chickens following 3-5 days post-infection. Based on the obtained results, it can be concluded that the developed AC-ELISA is able to detect H9, H1, and H3 influenza virus serotypes and is sufficiently sensitive and specific for the detection of infections caused by H9 serotype but, its applicability, sensitivity, and specificity for the detection of other serotypes of the virus remain to be determined.

Key words: influenza virus A; nucleoprotein; monoclonal antibody; antigen capture ELISA

Introduction

The genus A influenza viruses in the family *Orthomyxoviridae* cause significant economic losses in the poultry industry (1) as well as diseases in mammals, including horses, pigs, dogs, cows, and human beings (2). In recent years, several epidemics of influenza have appeared in human and animal populations. Due to frequent genetic alterations in the genome of the genus A influenza viruses (3), designing a universal hemagglutinin-(H) and/or neuraminidase- (N) based diagnostic method for these viruses is problematic. However, it has been shown that nucleoprotein (NP) is a highly conserved protein among different strains of genus A influenza viruses (3, 4, 5, 6, 7). Furthermore, the results of Glickman *et al.* (1995) revealed that monoclonal antibodies (Mabs) can bind with high affinity to NP and M (Matrix protein) proteins of influenza virus while hemagglutinin is not an appropriate candidate for virus detection, due to antigenic drifts (8). Therefore, the detection of NP in samples prepared from acute infections can be used as a diagnostic method for all types of the genus A influenza virus (9).

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Regarding the wide spread occurrence of the type H9N2 in Iran since 1998, as well as the probability of the appearance of other pathogenic types, such as H5N1, developing strategies in order to detect and control the infections is necessary (10).

The aim of this study was to design an Antigen Capture ELISA using a NP-specific monoclonal antibody for the diagnosis of infections caused by the genus A influenza viruses.

Material and methods

An NP-specific monoclonal antibody (D'C4 Mab), previously produced by Neisi *et al.* (11) in our laboratory, was used in this study. This Mab has been shown to react with H5, H7, and H9 serotypes of avian influenza viruses as well as H1 and H3 serotypes of human influenza viruses. To obtain an adequate amount of the Mab, D'C4 hybridoma (10^6 cells) was injected intra-peritoneally to three BALB/c mice. The mice have been previously treated with 0.5 ml of incomplete Freund's adjuvant through intra-peritoneal injection one week previously. The ascites fluids produced in these mice were harvested and centrifuged (10 min, 2000 rpm), and the supernatant was stored at -20 °C, as the Mab (1).

For production of the polyclonal antibody against NP, two female rabbits were intramuscularly injected with 100 µg of recombinant NP protein (previously produced in our laboratory by Jaidari *et al.*, (12) emulsified in ISA70 adjuvant at 0, 14, and 28 days. Ten days after the last injection, antibody production was evaluated using indirect ELISA, and the serum of the rabbit with the higher antibody titer was prepared and stored (13).

In the next step, AC-ELISA was developed as follows: 100 µl of diluted D'C4 ascites fluid (1:100 to 1:320000) in ELISA coating buffer ($NaHCO_3$ 84.01 g/mol, Na_2Co_3 103.99 g/mol, pH 9.6) was added to wells of an ELISA micro titer plate (SPL, Korea) and incubated overnight at 4 °C. Then the plate was washed 3 times with phosphate buffer saline (PBS) containing 0.05% Tween-20 (PBST). The free spaces were blocked using PBST plus 5% of skim milk for 3 hours at 37 °C. After washing, as previously mentioned, 100 µl of different dilutions of the recombinant NP were added to the columns of plate and incubated 2h at 37 °C. Washing was repeated and then a constant dilution of the Mab in PBST plus 2% skim milk was added to wells and incubated at room temperature for one hour. After three rinses, 100 µl of peroxidase conjugated anti-rabbit IgG (Sigma, USA), diluted in PBST plus 2% skim milk, was added to all wells and incubated for 30 minutes at room temperature. Washing was done four times, and then 100 µl of chromogen-substrate solution (Tetra methyl benzidine and H_2O_2 in acetate buffer) was loaded into all wells. The plate remained for 10 minutes in a dark room, and then reaction was stopped by adding 100 µl of 1 M HCl. Finally, the absorbance of wells was measured at 450 nm (4, 13).

To determine the sensitivity and specificity of the designed AC-ELISA, several experiments were performed. The specificity of the assay was evaluated by using different antigens, as follows: H9N2 serotype of avian Influenza virus (A/Chicken/Iran/AH-1/06), H3N2 and H1N1 human influenza viruses and also some avian viral and bacterial pathogens, including Avian Reovirus (vaccine strain, Merial, France), Infectious bronchitis virus (IBV, vaccine strain, Razi Institute, Iran), B1 and Lasota strains of Newcastle disease virus (NDV, vaccine strains, Razi Institute, Iran), Infectious bursal disease virus (IBDV, vaccine strain, Razi Institute, Iran), Infectious laryngotracheitis virus (ILTV, vaccine strain, Razi Institute, Iran), *Pasteurella multocida*, *Escherichia coli* and *Ornithobacterium rhinotracheale* (all clinical isolates), and *Mycoplasma synoviae* and *Mycoplasma gallisepticum* antigens (SOLEIL diagnostics- France).

In terms of sensitivity, the ability of AC-ELISA for the detection of A/Chicken/Iran/AH-1/06 (H9N2) influenza virus was compared to Hemagglutination assay (HA) and RT-PCR. For this purpose, 1:20 to 1:10240 dilutions of the virus were prepared and tested by HA and AC-ELISA, in triplicate. The allantoic fluid (AF) of an embryonated chicken egg was used as a negative control.

Different dilutions of the virus, i.e., 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:6000 and 1:7000 were also prepared for testing by RT-PCR. RNA was extracted using a High pure viral RNA kit (Takapouzist, Iran) and the cDNA was synthesized by using a commercial cDNA synthesis kit (kit, Takara, Kyoto) and H9 gene-specific forward primer (CTY CAC ACA GAR CAC AAT GG) (14). PCR reactions, performed by the H9 gene-specific forward and reverse (GTC ACA CTT

GTT GTT GTR TC) primers (14), contained 2 µl of cDNA, 10 picomols of each primer and 12.5 µl of 2X PCR Master Mix (Amplicon, Denmark), in a final volume of 25 µl. Thermal cycling parameters were as follows: an initial denaturation step (95 °C, 5 min); 35 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 40s) and extension (72 °C, 40 s); and a final elongation step (72 °C, 10 min).

Finally, the potential of the developed AC-ELISA for the diagnosis of avian influenza virus in clinical specimens was assessed by testing tracheal swabs collected from experimentally infected chickens. Five seronegative chickens were inoculated intra-ocularly and intranasally by 0.2 ml of 1:10 dilution of A/Chicken/Iran/A H-1/06 (H9N2) influenza virus and tracheal swabs were collected from 1 to 7 days after inoculation. The swabs were vortexed in 1 ml PBS and the released secretions were stored at -70 °C. Serum samples were also collected at 7th days and presence of antibody against the virus was investigated by Hemagglutination inhibition (HI) assay (13, 15). Tracheal swab samples were also prepared from 39 seronegative healthy chickens, as negative controls and for calculation of the cut-off point. The recombinant NP (with a concentration of 0.075 µg/ml) was applied as a positive control in this experiment. All samples were double tested in AC-ELISA and the mean absorbance of each swab was recorded. The optical density ratio of each swab sample to positive control (SP index) was calculated and the cut-off point was determined using the following formula:

Cut-off = mean SP values of seronegative healthy chickens + 3 SD

For comparison, 35 swabs collected during seven days from five experimentally infected chickens were also tested by RT-PCR, as described.

Results

In the checkerboard optimization of AC-ELISA, the optimal dilutions of the NP-specific monoclonal antibody, rabbit polyclonal antibody, and peroxidase conjugated anti-rabbit antibody were 1:5000, 1:1000 and 1:3000, respectively. Figure 1 shows the results of assessment of different antigens in AC-ELISA. As can be found, the absorbance of H1N1, H3N2, and H9N2 serotypes of influenza virus was approximately equal to the positive control (NP protein) while those of the other tested pathogens were equal to the negative control. These results confirm the high specificity of the designed AC-ELISA and the absence of false positive results (Figure 1).

The results of sensitivity assays have been presented in Table 1. As it suggests, the developed AC-ELISA can detect H9N2 influenza virus up to 0.1 HA unit. However, the sensitivity of the AC-ELISA was less than RT-PCR. The results of RT-PCR reaction revealed that an expected 488 bp Amplicon (a DNA segment of 488 bp) was produced in 1:100 to 1:6000 dilutions of the H9N2 virus in the allantoic fluid.

Optical densities of tracheal swabs from 39 uninfected healthy chickens in AC-ELISA were equal to PBS as the negative control (0.16), while the optical density was significantly higher for experimentally infected chickens, in some cases depending on the day of sampling, even greater than the OD value of the positive control (NP protein). Based on the calculated SP indices, the cut-off point of the designated AC-ELISA was 0.123. The SP indices of all samples prepared from infected chickens, 3 to 5 days after inoculation, were located above the cut-off line, i.e., were positive. Furthermore, some SP indices related to swabs collected between 2 and 6 days after experimental infection were also positive (not shown). To compare the AC-ELISA and RT-PCR, 35 tracheal swab samples from five experimentally infected chickens were also tested by RT-PCR. Following RT-PCR, it was confirmed that it was able to detect the H9 gene in swab samples collected from 2 to 6 days after infection and the most detections were 3 and 4 days after infection. OD values from experimentally infected chickens and the results of RT-PCR are shown in Table 2. At 7 days post-infection, all five experimentally infected chickens were positive in HI test, with the titers varying from 1:64 to 1:256.

Discussion

Rapid diagnosis of avian influenza plays a major role in the control of its epidemics. This subject is more prominent regarding the disease caused by highly pathogenic strains. Therefore, diagnosis using methods such as virus isolation, nucleic acid detection, and antigen detection is superior to serological methods (1, 9). Virus isolation is a time-consuming method and commonly has

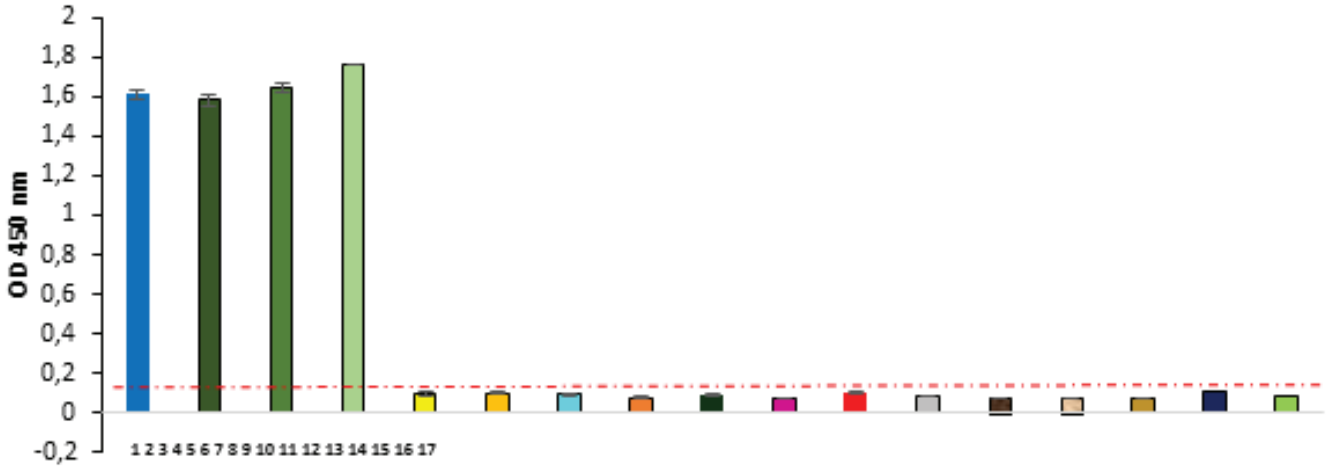

Figure 1: Results of cross-reaction of AC-ELISA with different viral and bacterial antigens.

1. Recombinant NP (0.075 µg/ml), 2. H9N2 1:160 dilution, 3. H1N1 1:30 dilution, 4. H3N2 1:30 dilution, 5. AF 1:30 dilution, 6. PBS 7. NDV B1 strains, 1:50 dilution, 8. (NDV Lasota strains, 1:50 dilution, 9. IBV, 1:50 dilution, 10. IBDV, 1:50 dilution, 11. ILTV, 1:50 dilution, 12. Avian Reovirus 1:50 dilution, 13. *Mycoplasma synoviae* 1:50 dilution, 14. *Mycoplasma gallisepticum* 1:50 dilution, 15. *Ornithobacterium rhinotracheale* (ORT) 1:50 dilution, 16. *Escherichia coli* 1:50 dilution, 17. *Pasteurella multocida* 1:50 dilution

Table 1: The sensitivity of AC-ELISA for H9N2 detection compare to HA titration and RT-PCR

dilution of virus	1:100	1:250	1:500	1:1000	1:2000	1:4000	1:5000	1:6000	1:7000
HA	+	+	+	–	–	–	–	–	–
AC-ELISA	+	+	+	+	+	+	+	–	–
RT-PCR	+	+	+	+	+	+	+	+	–

Table 2: Results (OD values) of AC-ELISA assay and RT-PCR on samples prepared from experimentally infected chickens

day	0	1	2	3	4	5	6	7
Inf. chick 1	0.112	0.117	0.408	2.33	2.46	1.01	0.17	0.165
Inf. chick 2	0.111	0.098	0.113	1.351	2.328	1.815	0.448	0.138
Inf. chick 3	0.127	0.136	0.205	0.782	2.445	2.426	0.182	0.174
Inf. chick 4	0.125	0.147	0.316	1.125	2.484	0.845	0.43	0.254
Inf. chick 5	0.11	0.113	0.57	2.241	1.928	0.741	0.229	0.125
RT-PCR	–	–	+	+	+	+	+	–

low sensitivity. RT-PCR is used for nucleic acid detection but despite its suitable specificity and sensitivity, in comparison with other diagnostic methods it is expensive, needs an expert operator, and is affected by contaminations, which leads to false positive results (1).

In contrast, antigen detection methods, especially AC-ELISA, have advantages such as rapid analysis of a large number of samples, desirable specificity and sensitivity, and lack of contamination risk in comparison to RT-PCR (4, 9).

Several Antigen Capture ELISA have been developed for the diagnosis of influenza viruses. Chomel *et al.* designed an immunocapture ELISA with 97% specificity and considerable sensitivity (15). In another study, the same researchers

subjected the nasal swabs of patients infected with H1N1 to immunocapture ELISA. The results revealed that this method can detect NP antigen in the nasopharyngeal swabs of infected humans even seven days after sampling (16). Ji *et al.* (13), using monoclonal and polyclonal antibodies specific for NP of H3N8, developed an AC-ELISA. Their results indicated that this AC-ELISA has sufficient sensitivity and specificity for the diagnosis of H3N8 and H7N1 strains of the equine influenza virus. Based on the report of Ji et al. (13), their designed AC-ELISA was able to find influenza viruses in nasal swabs prepared from experimentally infected horses from the 3rd to the 7th days after infection.

The investigations of Davison *et al.* (17), Gerentes *et al.* (18), Cattoli *et al.* (19), Wang *et al.* (20), He *et al.* (21), Velumani *et al.* (22), and Ho *et al.* (23) are examples of studies on developing AC-ELISA for the detection of all or specific serotypes of avian influenza viruses. Using two monoclonal antibodies, EB2-B3 and EB2-B5, specific for H6, Chen *et al.* (4) developed an AC-ELISA that was able to only detect H6 serotypes. He *et al.* (24) designed a dual function ELISA for the simultaneous detection of H7 and its specific antibody. This immunoassay had high sensitivity and 100% specificity for the diagnosis of H7 antigen and its specific antibody (24). Quan-wen et al. (1) produced recombinant H9 and its monoclonal antibody and then designed an AC-ELISA, which had 99.4% correlation, 97.1% sensitivity, and 94.4% specificity, in comparison with RT-PCR.

Due to the widespread prevalence of the H9N2 serotype of influenza virus in Iran and the risk of epidemics with H5 and H7 highly pathogenic serotypes, in the present study, an AC-ELISA based on NP and a NP-specific monoclonal antibody that can react to several serotypes of avian and human influenza viruses was designed. Following the optimization of principal variables, the sensitivity and specificity of this test for the detection of clinical infections caused by avian influenza virus were assessed. The developed AC-ELISA did not react with any other viral or bacterial antigens tested in this study and, therefore, its specificity was 100%.

In the study of Quan-wen *et al.* (1), the specificity of developed AC-ELISA, investigated using Egg drop syndrome virus, Infectious laryngotracheitis virus, Infectious bursal disease virus, Infectious bronchitis virus, and Newcastle disease virus, was estimated to be 94.4%.

The detection limit of the influenza virus with our developed AC-ELISA was as 0.1 HA unit. For comparison, the sensitivity of AC-ELISA in the study of Ho *et al.* (23) was estimated as 1-2 HA. Quan-wen *et al.* (1) also showed that the sensitivity of their developed AC-ELISA was limited to 8 HA. Therefore, it appears that the ELISA developed in our study is more sensitive than the hemagglutination assay was.

To compare it with the RT-PCR method, we also tested tracheal swabs collected from experimentally infected chickens by RT-PCR. Using RT-PCR, the virus was again detected most frequently between the 2nd and 6th days post-infection. Similar results have been found by Noroozian and Vasfi Marandi (25) that using RT-PCR detected H9 virus from 3 to 7 days after experimental infection.

Velumani et al. (22) also developed an AC-ELISA for H7N1 diagnosis and detected this virus in tracheal swab samples of experimentally infected chickens at the 3rd to 7th days after infection. They also used RT-PCR simultaneously and found that this method can detect the cDNA of the virus at the 3rd to 7th days after infection.

However, in the study of Chen *et al.* (4), the sensitivity of AC-ELISA for the H9 virus was 10^2 times less than that of RT-PCR.

The enhanced sensitivity of AC-ELISA in the present study can be related to the high affinity and avidity of the monoclonal and polyclonal antibodies, leading to an efficient antigen detection of the assay.

In the clinical assay, the introduced AC-ELISA was able to detect the influenza virus in swab samples prepared 3 to 5 days following experimental infection. Some experimentally infected chickens also had positive results at the 2nd to 6th days of experimental infection. The most significant reactions were obtained from samples collected four days after experimental infection. These results are in agreement with the report of Chomel *et al.* (15). They were able to detect a remarkable amount of NP antigen from the 2nd to 5th days, after infection and nearly in all cases, the most significant NP antigen detection was observed at the 4th day of infection. Lack of antigen detection in birds beyond the 5th to 6th days post infection can be due to immune responses which reduce the virus shedding. Because, as demonstrated, the HI titer of chickens was detected at the 7th day

post-infection, and this could be a reason for this finding.

While in the studies of Ji *et al.* (13) and Quan-wen *et al.* (1) the OD index was applied for determining positive or negative swab samples, in the present study the SP index was used. SP is a more accurate index for evaluating the positivity of clinical samples, because by applying this index the effects of possible variations on the outcomes of clinical samples, and positive control (NP antigen) will be eliminated.

With respect to the fact that the clinical experiment was based on the antigen detection in tracheal swabs, several tracheal swabs from uninfected chickens were also collected to determine the cut-off point. Chen *et al.* (4) and Chiu *et al.* (9) have used the mean OD values of negative controls plus 2 SD in order to calculate the cut-off point of their experiments. In the present study, the cut-off point was at first considered as the mean SP value obtained from the uninfected population plus 2 SD but it was consequently revealed that this cut-off point, despite increasing the sensitivity, will decrease specificity. Therefore, the cut-off point was set based on the mean SP value obtained from the uninfected population plus 3 SD.

In conclusion, the designed AC-ELISA has the potential for the detection of (at a minimum) the H9 Avian influenza virus and human H1 and H3 viruses. Further studies are necessary to verify the applicability of the assay for the detection of other influenza virus serotypes.

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RAZVOJ IN PREIZKUS NOVE METODE ELISA Z UJETJEM PROTITELES ZA ODKRIVANJE VIRUSA INFLUENCE A

M. Aria, M. R. Seifiabad Shapouri, M. Roayaei Ardakani, H. Motamedi, A. Yektaserasht, A. Jaidari

Povzetek: Namen raziskave je bil razvoj nove metode elisa z ujetjem protiteles za lažje diagnosticiranje okužbe z virusom ptičje influence. Nukleoprotein (NP) virusa smo najprej vezali z monoklonskimi protitelesi D'C4 in nato kompleks nukleoproteina in monoklonskega protitelesa označili s kunčjimi poliklonskimi protitelesi. Ta metoda ni kazala nobene navzkrižne (napačne) reakcije z drugimi virusi ali bakterijami ptičjega porekla, z njo pa smo lahko zaznali prisotnost virusa ptičje influence serotipa H9, pa tudi viruse človeške influence seroptipov H1 in H3. Občutljivost te metode za določanje linije H9N2 (A/Chicken/Iran/AH-1/106) virusa H9 je bila desetkrat višja v primerjavi z metodo hemaglutinacije in je bila primerljiva z občutljivostjo metode RT PCR. Z njo smo virus lahko določili v sapničnih izpirkih že 3 do 5 dni po okužbi poskusno okuženih piščancev. Iz pridobljenih rezultatov sklepamo, da nanovo razvita metoda elisa z ujetjem protiteles lahko zazna serotype H9, H1 in H3 virusa influence pri piščancih in ljudeh. Dokazali smo, da je metoda zanesljiva in zelo občutljiva za zaznavanje okužb s serotipom H9, uporabnost te nove metode za zaznavanje okužb z drugimi serotipi virusov influence pa bomo morali še dokazati.

Ključne besede: influenca virus A; nulleoprotein; monoklonska protitelesa; elisa z ujetjem protiteles

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Original Research Article

EFFECT OF *Garcinia cambogia* EXTRACT ON SERUM LEPTIN, GHRELIN, ADIPONECTIN AND INSULIN LEVELS AND BODY WEIGHTS IN RATS FED WITH HIGH LIPID DIET

Aysen Altiner[1], Isil Sahin[1], Tanay Bilal[2]*, Handan Aydin Vural[3]

[1]Department of Biochemistry, [2]Department of Animal Nutrition and Nutritional Diseases, [3]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Istanbul University, 34320 Avcilar, Istanbul, Turkey

*Corresponding author, E-mail: tanbilal@istanbul.edu.tr

Abstract: *Garcinia cambogia* (malabar tamarind, bitter kola) is seen abundantly in the evergreen forests of Konkan in South India. Hydroxycitric acid, mainly obtained from *Garcinia cambogia*, was identified by the end of the 1960's as a strong competitive inhibitor of the extramitochondrial enzyme adenosine triphosphate citrate lyase. The objective of this study was to present the effect of *Garcinia cambogia* extract known as weakener on the supportive or preventive hormones (leptin, ghrelin, adiponectin, insulin) of obesity in the rats fed the diet containing hydrogenated-vegetable oil and cholesterol. For this purpose, thirty female 5-6 months-old Sprague-Dawley rats were randomly assigned to three groups. Each group consisted of 10 rats. Group 1 (as control group) was fed with a basal diet while the diets of Groups 2 and 3 contained the hydrogenated-vegetable oil (20%) and cholesterol (1%) beside of other nutrients. *Garcinia cambogia* extract containing 6% hydroxycitric acid was added to the diet w/w of Group 3 after Day 30 up until Day 60. Blood samples were taken from animals on Days 0, 30 and 60 of the trial period. Body weights were weighed in the blood sampling days before sampling. Serum leptin, ghrelin, adiponectin, insulin levels and body weights were not significantly different between groups in the each sampling day. Serum leptin levels were significantly higher on Day 60 than on Days 0 and 30 in Groups 2 and 3. Serum ghrelin levels in Group 3 significantly decreased on Day 30 and significantly increased on Day 60. Serum adiponectin levels in Group 1 were significantly lower on Day 0 than on other days. The levels in Group 3 were significantly higher on Day 60 than on Day 0. The serum insulin levels and body weights were not significantly different between the days in each group. In conclusion, the high lipid diet in doses used in the study (20% hydrogenated-vegetable oil and 1% cholesterol) did not create fat accumulation or obesity in Sprague Dawley rats. Addition of *Garcinia cambogia* to diet (6%, *w/w*) of rats fed with the high lipid diet indicated no effect on weight gain. The evidences obtained from this research do not support the role of *Garcinia cambogia* plant spreadly used as a weight loss facilitator.

Key words: adiponectin; *Garcinia cambogia*; ghrelin; insulin; leptin

Introduction

Obesity is an inevitable consequence of chronic positive energy balance (1). Although genetic factors most certainly play an important role in having a tendency towards obesity, the real culprit is the environment leading to a sedentary lifestyle and excessive food consumption. Hypertension is a health problem included in or contributing to serious diseases such as type-2 diabetes, coronary artery disease and paralysis and is on the rise worldwide (2). Weight loss can usually be achieved with dieting, behavioral modification, and exercise as well as with pharmacotherapy or surgery. Unfortunately, body weight is usually rapidly regained after the termination of such interventions (3). Therefore, pharmacological and dietary treatments that might counteract overeating are of increasing interest (4).

Garcinia cambogia (malabar tamarind, bitter kola) is seen abundantly in the evergreen forests

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of Konkan in South India. Many traditional recipes in Kerala use it for its different flavor (5). Historically, *Garcinia cambogia* has been used by the Yoruba tribe in West Africa in the treatment of the respiratory tract infections such as throat ache and coughing. Preliminary studies carried out on *Garcinia cambogia* seeds have demonstrated their antifungal, anticancer, antihistamine, antiulcerogenic, antimicrobial and antiviral effects (6). Various *Garcinia* types are still being used in traditional medicine for the treatment of hepatitis, laryngitis and oral infections (7). Liquid extracts of the *Garcinia huillensis* tree bark are used against sexual diseases, wounds, bronchitis, pneumonia, angina, measles and dermatitis in traditional Zaire medicine (8).

Farombi et al. (9) reported that *Garcinia kola,* a powerful and natural antioxidant, may be used in the chemotherapeutic prevention of cancer and other diseases. Hydroxycitric acid, mainly obtained from *Garcinia cambogia* and *Garcinia indica* extracts, was identified by Watson and Lowenstein (10) towards the end of the 1960's as a strong competitive inhibitor of the extramitochondrial enzyme adenosine triphosphate citrate lyase. It is an enzyme catalyzing the division of citrate into acetyl-CoA and oxaloacetate, which is an important step in the adenosine triphosphate citrate lyase lipogenesis (11). Hydroxycitric acid that is the main acid in fruit and rind suppresses *de novo* fatty acid synthesis by inhibiting this enzyme, and increases the rates of hepatic glycogen synthesis (12). Hydroxycitric acid also decreases the hyperglycemic and hyperinsulinaemic responses to oral or intragastric glucose loads (13).

Leonhardt et al. (14) reported that substances that block the fatty acid synthesis might be useful to prevent the body weight gain because weight gain in adults, and in particular weight regain after the body weight loss, usually comprises a buildup of fat. Researchers emphasized that hydroxycitric acid may be most effective in decreasing the food intake and preventing the body weight gain when the energy intake exceeds energy expenditure and the fatty acid synthesis is increased.

Adipose tissue plays an active role in regulation of the energy balance and nutrient metabolism (15). This tissue synthesizes and secretes a number of cytokine hormones that are involved in the regulation of energy homeostasis, insulin action, and lipid metabolism. Leptin and adiponectin are the adipose tissue-specific factors. The variation in these hormones may indicate the recovery in metabolic and cardiovascular risk profile after weight loss. Adiponectin, leptin and ghrelin are all involved in the regulation of energy homeostasis, the obesity and the moderate weight loss (1). Leptin has direct effect on some cell types including liver, bone and platelets, *in vitro* (16). While the amount of fat in body is responsible for 50-60% of plasma leptin, the factors such as age, gender, diurnal variation, hormones (primarily insulin) and cytokines (mainly TNF-α) are also effective on plasma leptin levels. Hyperleptinemia is known to be a component of metabolic syndrome (17).

Loss of fat mass causes increase of the circulating ghrelin levels, since ghrelin is a strong orexigenic signal (18). Plasma ghrelin levels in obese individuals have been found to be lower than those of thin individuals in the control group (19). Adiponectin in humans has been reported as a good marker of metabolic health (20). Adiponectin has sensitive to insulin, anti-atherosclerotic and anti-inflammatory properties (21). Weight loss induced by especially low-carbohydrate diet has been reported to increase the adiponectin concentrations in humans (22). Blood adiponectin concentrations in humans are negatively correlated with the abdominal fat mass and adipose tissue size (23).

Objective of this study was to determine the effect of *Garcinia cambogia* extract known as weakener on the supportive or preventive hormones (leptin, ghrelin, adiponectin, insulin) of obesity in rats fed the diet containing hydrogenated-vegetable oil and cholesterol.

Materials and methods

The study was approved by the Ethical Committee of Istanbul University (No:62/29042010), and was carried out according to the legal requirements of the relevant national authority. Thirty female 5-6 months-old Sprague-Dawley rats (pathogen-free) were used in the study. They were housed individually to standard cages (33x23x12 cm) under the controlled conditions of temperature, lighting and humidity. Rats were randomly assigned to the three experimental groups of 10 animals each. The tap water and the appropriate diets of all groups (Table 1) were given as *ad libitum* from the beginning up until the end of trial period (Day 60). *Garcinia cambogia* fruit rind

extract was provided by General Nutrition Products (General Nutrition Products, Inc., SC, USA). Group 1 (as control group) was fed with a basal diet while the diets of Groups 2 and 3 contained the hydrogenated-vegetable oil (20%) and cholesterol (1%) beside of other nutrients. *Garcinia cambogia* extract containing 6% hydroxycitric acid was added to diet (*w/w*) of Group 3 after Day 30 up until Day 60. Body weights were weighed in the blood sampling days before sampling.

Blood samples were taken from all animals through coccygeal venipuncture on Days 0, 30 and 60 of the trial period after the fasting overnight and after the anesthetized with Ketamine-HCL (100 mg/kg i.p.). Serums were separated by centrifugation at 3000 rpm for 20 minutes, and stored at – 86 °C until analysis. Serum leptin, ghrelin, adiponectin and insulin analyses were done by using commercially available kits with ELISA equipment (μQuant, Bio-Tek). Serum leptin levels were determined by RayBio Rat Leptin ELISA kit (ELR-Leptin-001, RayBiotech Inc., Norcross GA 30092, USA). Serum ghrelin levels were determined by Rat Ghrelin ELISA kit (E90991Ra, USCN Life Science Inc., Houston, TX 77082, USA). Serum adiponectin levels were determined by AssayMax Rat Adiponectin ELISA kit (ERA2500-1, AssayPro, MO 63304, USA). Serum insulin levels were determined by Rat Insulin ELISA kit (CSB- E05070r, Cusabio Biotech, Wuhan, Hubei Province 430223, P.R.China).

Results were presented as mean ± standard error. Data were compared at the significance level of $p \leq 0.05$ by using the one way analysis of variance (Tukey's multiple range test) between the groups on each blood sampling day, and between the days within each group. All statistical analyses were performed by using the software package program (SPSS for windows, Standard version 10.0, 1999, SPSS Inc., Headquarters, Chicago, IL, USA).

Results

The comparisons between the groups and between the days were indicated in Figures. Serum leptin, ghrelin, adiponectin, insulin levels and body weights were not significantly different between groups in the each sampling day. Serum

Table 1: Composition of experimental diets

Composition of nutrients (%)	**Group 1**	**Group 2**	**Group 3**
Cracked barley	23	10	4.5
Fish meal	2	3	4
Cracked wheat	42.5	30.5	40.5
Garcinia cambogia extract	-	-	6
Hydrogenated vegetable oil	-	20	20
Cholesterol	-	1	1
Rasmol	15	15	3.5
Liquid vegetable oil	2	2	2
Soybean meal	14.5	17.5	17.5
Vitamin-mineral mixture	1	1	1
Calculation of nutrients			
Crude Protein (%)	19.5	19.5	19.5
Metabolisable energy (MJ/kg)	12.5	13.7	13.7

Vitamin-mineral mixture, kg: Vitamin A 12.000 U, Vitamin D_3 1.500 U, Vitamin E 104 U, Vitamin K 15 mg, Vitamin B_1 14 mg, Vitamin B_2 11 mg, Vitamin B_6 14 mg, Vitamin B_{12} 20 mg, Folic acid 2.5 mg, nicotinic acid 78 mg, Pantothenic acid 26 mg, Biotin 334 μg, Choline chloride 1635 mg, Selenium 0.36 mg, Cobalt 0.46 mg, Iodine 1.41 mg, Zinc 95 mg, Manganese 68 mg, Copper 20 mg, Iron 104 mg

Group 1: Control group

Group 2: Group fed with the high lipid diet

Group 3: Group fed with the high lipid diet and *Garcinia cambogia* extract

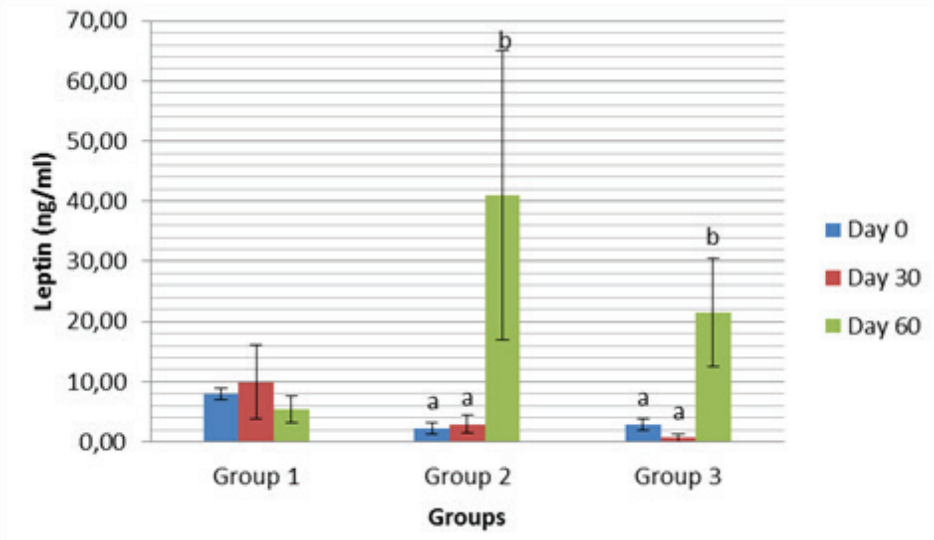

Figure 1: Serum leptin levels in rats fed with the high lipid diet

a,b: Different superscripts indicate significant differences between days ($p \leq 0.05$)
Mean ± standard error
Group 1: Control group
Group 2: Group fed with the high lipid diet
Group 3: Group fed with the high lipid diet and *Garcinia cambogia* extract

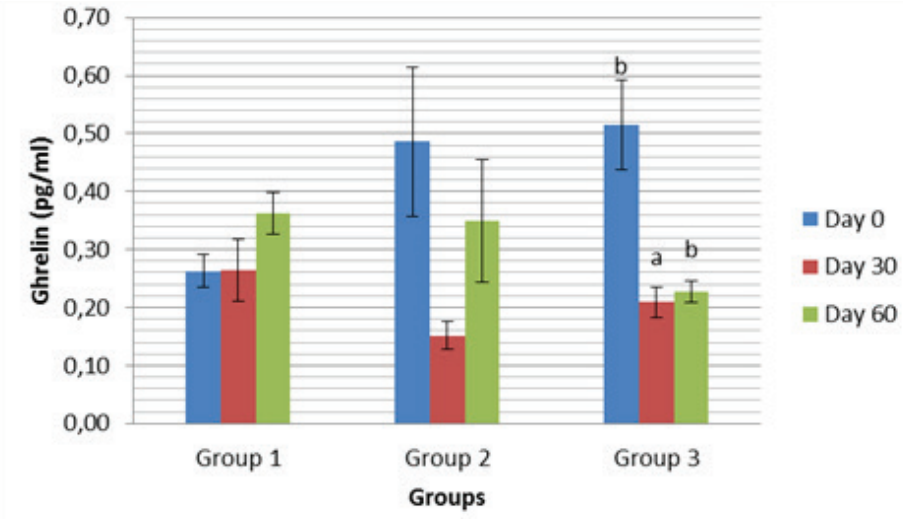

Figure 2: Serum ghrelin levels in rats fed with the high lipid diet

a,b: Different superscripts indicate significant differences between days ($p \leq 0.05$)
Mean ± standard error
Group 1: Control group
Group 2: Group fed with the high lipid diet
Group 3: Group fed with the high lipid diet and *Garcinia cambogia* extract

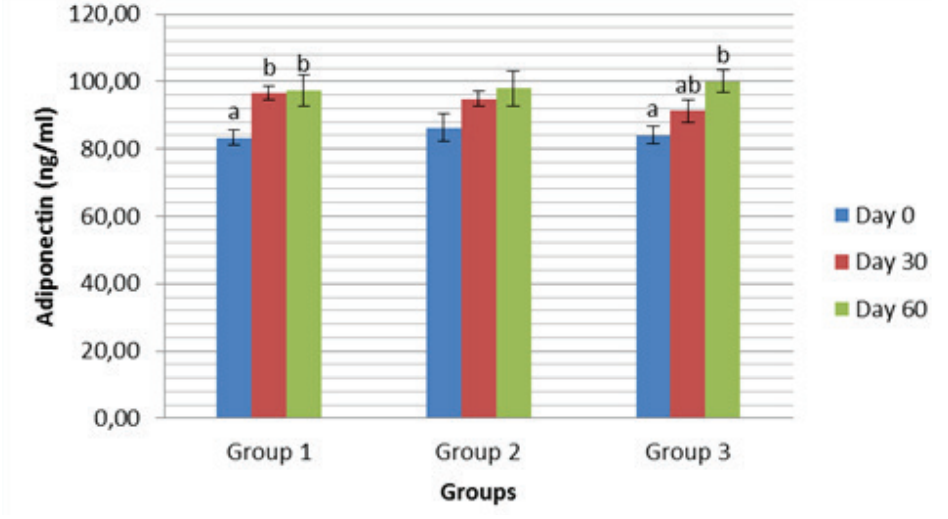

Figure 3: Serum adiponectin levels in rats fed with the high lipid diet

a,b: Different superscripts indicate significant differences between days ($p \leq 0.05$)
Mean ± standard error
Group 1: Control group
Group 2: Group fed with the high lipid diet
Group 3: Group fed with the high lipid diet and *Garcinia cambogia* extract

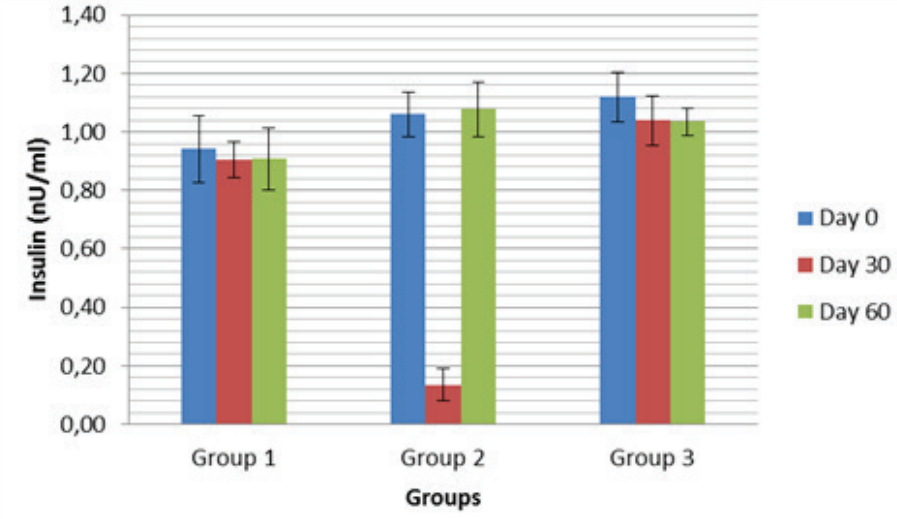

Figure 4: Serum insulin levels in rats fed with the high lipid diet

Mean ± standard error
Group 1: Control group
Group 2: Group fed with the high lipid diet
Group 3: Group fed with the high lipid diet and *Garcinia cambogia* extract

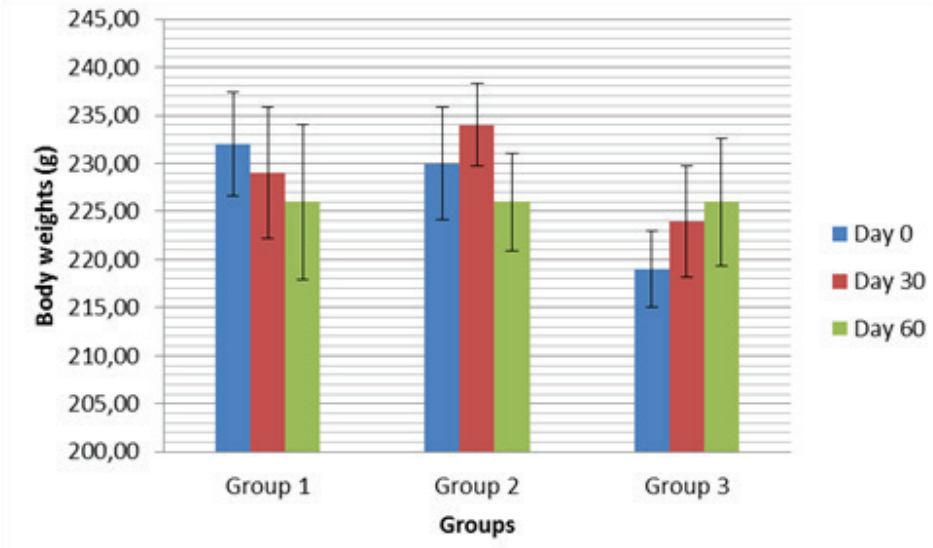

Figure 5: Body weights in rats fed with the high lipid diet

Mean ± standard error
Group 1: Control group
Group 2: Group fed with the high lipid diet
Group 3: Group fed with the high lipid diet and *Garcinia cambogia* extract

Table 2: The correlations between serum indices

Indices		Adiponectin	Leptin	Ghrelin
	r	-0.083		
Leptin	p	0.518	-	-
	n	63		
	r	-0.101	+0.022	
Ghrelin	p	0.118	0.868	-
	n	58	58	
	r	+0.012	-0.173	-0.011
Insulin	p	0.922	0.174	0.936
	n	74	63	58

r: Correlation coefficient
p: Significance level
n: Total sample number

leptin levels were significantly higher on Day 60 than on Days 0 and 30 in Groups 2 and 3. Serum ghrelin levels in Group 3 significantly decreased on Day 30 and significantly increased on Day 60. However, they were not significantly different between Days 0 and 60. Serum adiponectin levels in Group 1 were significantly lower on Day 0 than on other days. They increased with the advancing days in Group 3, and they were significantly higher on Day 60 than on Day 0. The serum insulin levels and body weights were not significantly different between the days in each group. The correlations between serum indices were indicated in Table 2. No positive or negative significant correlations were determined between the total serum leptin, ghrelin, adiponectin and insulin levels.

Discussion

Preuss et al. (24) gave 4677 mg/day hydroxycitric acid to subjects for 8 weeks, and determined that serum leptin levels gradually significantly decreased towards the end of 8 weeks. Gatta et al. (25) gave 2 g hydroxycitric acid with lunch to the male subjects and took the regular blood samples until the evening meal. They found the mean plasma leptin concentration to be 3.24 ng/ml in controls and 3.72 ng/ml in the hydroxycitric acid group. The researchers did not find a significant difference between two groups. Saito et al. (26) determined the leptin levels in Zucker obese rats, as 63.2 ng/ml in the control group and as 68.9 ng/ml in the experimental group that is given 154 mmol/kg hydroxycitric acid. The researchers reported that leptin did not mediate the unappetizing effect of hydroxycitric acid, and there was no significant difference regarding the serum leptin concentrations between the experimental groups.

Hayamizu et al. (27) reported that no statistically significant difference was found between experimental and control groups in the study where mice were given 3.3% *Garcinia cambogia* with water containing 10% saccharose for 4 weeks. The researchers determined leptin levels as 11.7 ng/ml in the control group and 5.8 ng/ml in the experimental group. They reported that *Garcinia cambogia* administration decreased the serum leptin levels and the leptin/white adipose tissue ratio without producing any changes in the appetite although leptin is a hormone with appetite controlling activity. In the present study, serum leptin levels were not significantly different between all groups. They were significantly higher on Day 60 than on Days 0 and 30 in Groups 2 and 3. This significant rising of serum leptin concentrations in Groups 2 and 3 on Day 60 were due to the fatty feeding because fatty feeding increases leptin synthesis (28). The fact that there is no significant difference between control and experimental groups is similar to the findings of Gatta et al. (25), Saito et al. (26) and Hayamizu et al. (27).

Faraj et al. (1) determined fairly low plasma ghrelin levels in subjects with morbid obesity. Cummings et al. (29) reported that plasma ghrelin levels increase after weight loss induced by diet. Gatta et al. (25) determined the mean plasma ghrelin level to be 1.11 ng/ml both in the control group and in the male subjects taking 2 g hydroxycitric acid with lunch. In the present research, no significant difference in serum ghrelin levels was found between groups. The feeding with the high lipid diet did not lead to significant changes in serum ghrelin levels in the study. At the same time, the addition of *Garcinia cambogia* to food did not cause any significant change in serum ghrelin levels and this is similar to the findings of Gatta et al. (25).

Gatta et al. (25) determined plasma insulin concentration as 18.3 μU/ml in the control group and as 18.64 μU/ml in the subjects taking 2 g hydroxycitric acid with lunch, and they could not find a significant difference between 2 groups. In the present study serum insulin concentrations were not significantly different between the groups in different days or between the days within each group. The finding that hydroxycitric acid has no effect on serum insulin levels was similar to the findings of Hayamizu et al. (30), Leonhardt et al. (31) and Gatta et al. (25).

Faraj et al. (1) reported that adiponectin is not a parameter associated with weight. Engström et al. (32) reported that adiponectin is indirectly proportional to insulin. In the study, serum adiponectin levels were not significantly different between 3 groups, and the levels significantly increased with the advancing days in Group 3. The levels were significantly higher on Day 30 than on Day 0 in the control group. It was not encountered any literature similar to the subject of this study as a result of the comprehensive literature searches.

Koshy et al. (5), added 1 mg/100 g/day

of *Garcinia cambogia* flavonoids to the diets containing cholesterol, and did not observe significant changes in weight gain in the experimental animals compared to the control animals during 90 day duration of the study. Farombi et al. (9) added 200 mg/kg body weight/day of kolaviron being a seed extract of *Garcinia kola* to the diet of rats for a week, and did not determine a significant change in the body weights compared to control group. The feeding with the high lipid diet or the high lipid diet and *Garcinia cambogia* extract did not cause the significant differences in the body weights of rats in the present study. Also, the significant changes did not occur in the body weights of rats in all groups with the advancing days. These findings are in consistent with the findings of Koshy et al. (5) and Farombi et al. (9).

In conclusion, the high lipid diet in doses used in the study (20% hydrogenated-vegetable oil and 1% cholesterol) did not create fat accumulation or obesity in Sprague Dawley rats. Addition of *Garcinia cambogia* to diet (6%, *w/w*) of rats fed with the high lipid diet indicated no effect on weight gain. The differences in the dose and effectiveness of *Garcinia cambogia* extract used in this study could be reason for the opposite findings obtained in the present study compared to the findings obtained in other some studies. For example, hydroxycitric acid that is an active ingredient of *Garcinia cambogia* can be found sometimes in the free acid form and sometimes in the lactone form, and it is thought to be the biologically active inhibitor of adenosine triphosphate citrate lyase of only free acid form (33). As a result, the evidences obtained from this research do not support the role of *Garcinia cambogia* plant spreadly used as a weight loss facilitator.

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Authors declare that there are no known conflicts of interest associated with this publication and that there has been no significant financial support for this work that could have influenced its outcome.

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VPLIV IZVLEČKA RASTLINE *Garcinia cambogia* NA RAVEN LEPTINA, GRELINA, ADIPONEKTINA IN INZULINA TER NA TELESNO TEŽO PRI PODGANAH, HRANJENIH S HRANO Z VISOKO VSEBNOSTJO MAŠČOB

A. Altiner, I. Sahin, T. Bilal, H. Aydin Vural

Povzetek: *Garcinia cambogia* je pogosta rastlina v tropskih gozdovih južne Indije. Hidroksicitronska kislina, ki je v velikih količinah v rastlini *Garcinia cambogia*, je bila v šestdesetih letih prejšnjega stoletja prepoznana kot močan kompetitivni zaviralec zunajmitohondrijskega encima ATP-citratna liaza. Namen raziskave je bil ugotoviti, ali izvleček rastline *Garcinia cambogia* lahko vpliva na izločanje hormonov, ki sodelujejo pri urejanjeu telesne teže (leptin, grelin, adiponektin, inzulin) pri podganah, hranjenih s hrano z visoko vsebnostjo maščob (rastlinska olja in holesterol). V raziskavo je bilo vključenih 30 podganjih samic seva Sprague-Dawley, starih 5 do 6 mesecev, ki so bile naključno razporejene v eno od treh poskusnih skupin. Podgane iz prve skupine so prejemale navadno hrano, podgane iz druge skupine pa hrano z dodanimi 20 % rastlinskih olj ter 1 % holesterola. Izvleček *Garcinie cambogie* je bil dodajan v hrano podganam iz tretje skupine, tako da so prejemale v hrani 6 % hidroksicitronske kisline. Podgane so bile v poskusu 60 dni, s tem da so živali iz tretje skupine prejemale v hrani izvleček *Garcinie cambogie* od 30. do 60. dneva poskusa. Podganam smo odvzeli kri 1., 30. in 60. dan poskusa, ko so bile tudi stehtane. Raven letpina je bila v skupinah 2 in 3 statistično značilno višja na 60. dan v primerjavi s 1. in 30. dnevom. Raven grelina je bila v skipini 3 30. dan poskusa statistično značilno nižja, 60. dan pa statistično značilno višja. Raven adiponektina je bila v skupini 1 statistično značilno višja 30. in 60. dan poskusa. Raven inzulina ter telesna teča pa se nista razlikovali med skupinami v nobeni časovni točki. Ugotovimo lahko, da hrana z visoko vsebnostjo maščob ni povzročila v časovnem okviru raziskave debelosti, prav tako pa izvleček rastline *Garicnia cambogia* ni vplival na telesno tečo ali na raven hormonov in torej ni primeren kandidat za naravno zdravilo za pomoč pri hujšanju.

Ključne besede: adiponectin; *Garcinia cambogia*; ghrelin; insulin; leptin.

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Original Research Article

EFFECTS OF BEE POLLEN AND PROPOLIS ON PERFORMANCE, MORTALITY, AND SOME HAEMATOLOGICAL BLOOD PARAMETERS IN BROILER CHICKENS

Ivana Klarić[1], Matija Domaćinović[1], Vatroslav Šerić[2], Ivan Miškulin[2]*, Mirela Pavić[3], Ksenija Paradinović[2]

[1]Faculty of Agriculture, Josip Juraj Strossmayer University of Osijek, Vladimira Preloga 1, [2]Faculty of Medicine, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 10E, 31000 Osijek, [3]Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia

*Corresponding author: E-mail: ivan.miskulin@mefos.hr

Abstract: This experimental study aimed to determine how propolis and bee pollen (each supplement separately or in combination in a certain proportion), as additives to broiler feed, affect performance, mortality and the values of the selected haematological blood parameters in chickens. This experimental study was conducted on 200 Ross 308 chickens of equally distributed sex, which were randomly divided into five groups. Throughout the whole study, the control group of chickens was fed ordinary feed mixture, while the feed mixture that was fed to the experimental groups of chickens contained propolis and/or bee pollen. The average values of body weight of chickens were significantly higher on the 1st (P=0.001), 2nd-5th (P<0.001) and 6th (P=0.002) weeks of fattening in the experimental groups of chickens in comparison to the control group; the average values of weight gain of chickens were significantly higher on the 1st (P<0.001), 2nd (P=0.002), 3rd (P<0.001), 4th (P=0.029) and 5th (P=0.009) weeks. Lower mortality was recorded in all the experimental groups in comparison to the control group of chickens (P=0.031). Higher values of MCV (P=0.009) and haematocrit (P=0.015) and lower values of the leukocyte count (P=0.029) and of the relative ratio of Mo (P<0.001) were recorded in the experimental groups of chickens in comparison to the control group on the 21st day of fattening. Higher values of the relative ratio of heterophils (P<0.001) and lower values of the relative ratios of lymphocytes (P<0.001) and monocytes (P=0.027) were recorded in the experimental groups of chickens in comparison to the control group on the 42nd day of fattening. The results of this study showed that supplementation with propolis and/or bee pollen improved the general health condition of the chickens and positively affected the performance and the values of the selected haematological blood parameters in the chickens.

Key words: natural feeding additives; chickens; health; blood; chicken feeding

Introduction

Sub-therapeutic doses of antibiotics were often used in livestock breeding to improve production indicators and animal health, but also to control pathogens effectively. However, due to the negative effects of antibiotics, such as antibiotic resistance and the presence of antibiotic residues in the final product, the European Commission banned the use of antibiotics as growth agents in 2006 (1,2). This concern has led many researchers to investigate and look for alternatives to promote growth, including the use of natural supplements as feed additives, which would have positive effects both on the growth of chickens and feed utilization. The latter is also important to please consumers' increased demands for the usage of natural products as alternative additives in foods (3). In this context, research has been done on the use of probiotics, prebiotics, antioxidants, acidifiers, enzymes and various plant products as additives in broiler feeding. Recently, propolis and bee pollen have also been considered as potential new additives (1,2,4).

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Propolis is a natural resinous bee product (5). It is composed of more than 50% lipophilic substances of leaves, plant resins and balsams, plant latex and vegetable glue. It is approximately 30% waxes, 10% essential and aromatic oils, 5% pollen, and the remaining 5% is a mixture of different substances such as polyphenolic substances, e.g. flavonoids, organic phenols, ketones and terpenes, as well as organic debris, i.e. wood fragments (6). Moreover, propolis contains minerals, such as Mg, Ca, K, Na, Cu, Zn, Mn and Fe, vitamins B_1, B_2, B_6, C and E, as well as fatty acids and some enzymes (7). Its biological activity depends on the active substances of a polyphenolic fraction, flavonoids for the most part, but also on aromatic acids, esters of phenolic acids, triterpenes, lignans and the like (8). These bioactive components of propolis are responsible for antibacterial, antiviral, antifungal, antiprotozoal, antimicrobial, analgesic, anti-inflammatory, antioxidant, locally anaesthetic, cytostatic, i.e. anticancer, as well as the immunostimulating and immunomodulatory effects of propolis both in humans and animals (9,10).

Bee pollen consists of the male gametophytes of seed plants (11,12). To date, about 250 various chemical compounds have been detected in it, including carbohydrates, fats, proteins, vitamins, macro- and microelements, antibiotics (inhibins), hormones, enzymes, organic acids, essential oils, rutin, and others (11). Bees collect pollen from flowers and mix it with their own digestive enzymes (13). Bee pollen is rich in proteins (25%) and essential amino acids. Moreover, it contains 6% of oils, 51% of which are polyunsaturated fatty acids; 39% of polyunsaturated fatty acids are linolenic acid, 20% palmitic and 13% linoleic acid. Bee pollen contains more than 12 vitamins (B-complex vitamins, vitamins A, C, D, E and K_3), 28 minerals, 11 enzymes or coenzymes and 11 different carbohydrates, which comprise 35-61% of pollen. Carbohydrates are mainly glucose and fructose. As previously mentioned, bee pollen also contains phytochemicals; flavonoids, carotenoids, terpenes, phytosterols, and polyphenols are the most important among them (13,14). The bioactive compounds of bee pollen include flavonoids, phenolic acids and their derivatives that are responsible for the bactericidal, antiviral, antifungal, analgesic, anti-inflammatory, antioxidant, immunostimulating and immunomodulatory effects of these substances in humans and animals (11,15).

This study aims to determine how propolis and bee pollen affect performance, mortality and the values of the selected haematological blood parameters in broiler chickens.

Materials and methods

Animals and diets

The study included a total of 200 day-old chickens of the Ross 308 provenance (16). The fattening trial of the chickens was carried out on a family farm in eastern Croatia under the supervision of the Department of Nutrition, Anatomy and Physiology of Domestic Animals, Faculty of Agriculture, University of Osijek. The study was approved by the Ethics Committee of the Faculty of Agriculture in Osijek, Josip Juraj Strossmayer University of Osijek. The total of 200 chickens of the Ross 308 provenance, evenly distributed sexes, were randomly divided into 5 groups (40 chickens in each group), one of which was the control group (K) and the other four experimental groups (P1, P2, P3, P4). For the purpose of more effective monitoring of all the investigated indicators, on the seventh day of the trial, all the chickens were marked with leg rings.

During the study, all the groups of chickens were fattened under the same conditions. Temperature, humidity and lighting in the facility were maintained within optimum limits according to the manufacturer's recommendations for the Ross 308 hybrid. Fattening was conducted on wooden sawdust and lasted for 6 weeks (42 days). During the study, feed and water were given to chickens' ad libitum.

From days 1-21 of the study, chickens were fed a mixture of starter; from days 22-42 of the study, chickens were fed a mixture of finisher. The composition and calculated analysis of feed mixtures used in the chickens fattening are shown in Table 1. Throughout the study the control group (K) of chickens was fed a standard feed mixture without additives, while the experimental groups of chickens (P1, P2, P3, P4) were fed feed mixtures that contained additives – propolis and/or bee pollen as follows: P1 group: feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 group: feed mixture + 0.5 g of propolis/kg of feed mixture; P3 group: feed mixture + 1.0 g of propolis/kg of feed mixture;

Table 1: The composition and calculated analysis of feed mixtures used in the chicken fattening

Fodders, %	Starter	Finisher
	day 1–21	day 22–42
Corn grain	45.00	46.10
Flour middling	2.80	3.00
Dehydrated alfalfa	2.80	4.00
Soybean meal	20.20	10.00
Sunflower meal	4.00	4.00
Yeast	4.00	3.00
Full fat soybean	12.40	20.00
Vegetable oil	3.70	5.00
Monocalcium phosphate	1.20	1.20
Limestone	1.60	1.40
Salt	0.30	0.30
Premix*	1.00	1.00
Pigozen 801	1.00	1.00
Total	100.00	100.00
Calculated analysis		
Crude protein, %	21.02	19.15
Crude fat, %	8.36	10.96
Crude fibre, %	4.96	5.05
Lysine, %	1.11	0.96
Methionine, %	0.66	0.61
Tryptophan, %	0.26	0.23
Calcium, %	1.04	0.98
Phosphorous, %	0.70	0.67
ME, MJ/kg	12.30	13.10

**Each 1 kg of premix contained: Vitamin A 1200000 IU; Vitamin D3 200000 IU; Vitamin E 3000 mg; Vitamin K3 250 mg; Vitamin B1 150 mg; Vitamin B2 600 mg; Vitamin B6 200 mg; Vitamin B12 1 mg; Folic acid 50 mg; Niacin 4400 mg; Ca Panthothenate 1500 mg; Biotin 10mg; Choline chloride 50000 mg; Iron 5000 mg; Copper 700 mg; Manganese 8000 mg; Zinc 5000 mg; Iodine 75 mg; Cobalt 20 mg; Magnesium 750 mg; Selenium 15 mg; Antioxidant BHT 10000 mg; Methionine 100000 mg; Herbal carrier 1000 g.*

P4 group: feed mixture + 20 g of bee pollen/kg of feed mixture. Blending of propolis and bee pollen into the feed mixture was performed in a vertical mixer, and administration of propolis and bee pollen started from the first day of the trial.

Performance

Individual body weight (BW) of each chicken was measured on the 1st, 7th, 14th, 21st, 28th, 35th, and 42nd days of fattening period using an Avery Berkel FX 220 electronic scale. Based on the measured values, the average value of the body weight of chickens from all the groups has been calculated, while the difference between body weights served for the calculation of weight gains (WG). During the fattening period, feed consumption (FC) was recorded at weekly intervals for each group of chickens. Based on the total amount of consumed feed and overall weight gain, a feed conversion ratio (FCR) was calculated for the periods between weeks 1-3, 3-6, and for the overall experiment (weeks 1-6).

Blood sample collection and analysis

For the entire duration of the study, the mortality of chickens was monitored and recorded on a weekly basis. Blood sampling was performed twice during the study period (on days 21 and 42 of the study), on randomly selected chickens (10 birds from each group). Chickens that were selected for blood sampling on day 21 were used as experimental animals for the monitoring of all the investigated parameters until the end of the study. Blood sampling was performed by the puncture of the wing vein (lat. *v. cutanea ulnaris*) with direct needle injection coupled with a test tube under vacuum. The collected blood samples were analysed for the following haematological parameters (red blood cells (RBC) or erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC) or leukocyte count, differential blood count). Total number of erythrocytes (10^{12}/l), as well as the values of haemoglobin (g/l), haematocrit (L/l), MCV (fL; 1fL=10^{-15}L), MCH (pg; 1pg=10^{-12}g) and MCHC (g/L) were determined using CELL-DYN 1700 automatic analyser (Abbott Diagnostics, USA). A haemocytometer was used to count the total number of leukocytes (10^{9}/l) in the whole blood. The amounts of specific leukocyte types (heterophils-He, lymphocytes-Ly, eosinophils-Eo, monocytes-Mo and basophils-Ba) were counted in blood smear using May-Grünwald and Giemsa staining by means of an Olympus CH2O microscope. Laboratory analysis of all the abovementioned blood parameters of broilers was performed at the Department of Clinical Laboratory Diagnostics, Clinical Hospital Centre Osijek. The Wakenell reference values (17) were used to interpret the obtained results of the analysis of the haematological blood parameters of broilers.

Statistical analysis

Upon confirming normality of data distribution with a Shapiro-Wilkinson test, all data were processed by the methods of descriptive statistics. The numerical variables were described as the mean and standard deviations. The ANOVA and Kruskal-Wallis test were used for the comparison of numerical variables among the groups. The categorical variables were described in absolute and relative frequencies. Fisher's exact test was used for the comparison of categorical variables between the groups. The level of statistical significance was set at $P<0.05$. Statistical analysis was done using the Statistica for Windows 2010 statistical package (version 10.0, StatSoft Inc., Tulsa, OK). Different lowercase letters at the level of statistical significance of $P<0.05$ assigned to the individual values in the tables indicate a statistically significant difference, while the same lowercase letters assigned to certain values in the tables indicate the absence or lack of statistically significant differences.

Results and discussion

The average values of the measured body weights of chickens from all the groups according to the fattening period are shown in Table 2.

Statistical analysis has shown that there was a statistically significant difference in the average body weights of chickens between the experimental groups and the control group on the 7th, 14th, 21st, 28th, 35th and 42nd days of the fattening period.

The average values of the calculated weight gains of chickens from all the groups according to the fattening period are shown in Table 3.

Statistical analysis has shown that there was a statistically significant difference in the average weight gain of chickens between the experimental groups and the control group on the 1st, 2nd, 3rd, 4th and 5th weeks of the fattening period.

Considering the feed conversion ratio by groups of chickens and fattening periods the study revealed several differences. In the period from the 1st to 3rd weeks of fattening, the lowest feed conversion ratio (1.69) was recorded in the P4 group, and the highest (1.93) in the control group of chickens. In the period from 4th to 6th weeks of fattening, the lowest feed conversion ratio (2.38) was recorded in the P1 group, and the highest (2.52) in the P2 group of chickens. During the whole fattening period (from 1st to 6th weeks of fattening), the lowest feed conversion ratio (2.19) was recorded in the P1 and P4 groups, and the highest (2.31) in the P2 group of chickens.

The overall mortality rate was found to be 3.5% (7/200) during the study. The highest mortality rate of 10% (4/40) was recorded in the

Table 2: Body weights of chickens according to the fattening period (g)

Days	Statistical parameters	Group of chickens					*P – value
		K	P1	P2	P3	P4	
1st	$\bar{x}$	41.23	41.25	41.30	41.25	41.23	0.999
	s	1.40	1.63	1.65	1.66	1,51	
7th	$\bar{x}$	125.95^{a}	129.79^{ab}	131.95^{ab}	135.59^{b}	141.85^{c}	0.001
	s	18.39	17.05	14.61	15.17	17.43	
14th	$\bar{x}$	303.89^{ac}	307.13^{a}	324.50^{cd}	341.23^{bd}	352.36^{b}	<0.001
	s	62.97	43.84	47.09	39.74	40.36	
21st	$\bar{x}$	607.67^{a}	655.51^{b}	670.33^{b}	719.90^{c}	743.46^{c}	<0.001
	s	112.42	93.47	95.86	84.90	82.16	
28th	$\bar{x}$	1018.03^{a}	1077.10^{ad}	1106.30^{bd}	1140.51^{b}	1187.13^{c}	<0.001
	s	173.25	138.90	154.14	103.76	120.58	
35th	$\bar{x}$	1526.03^{a}	1581.15^{ab}	1599.95^{ab}	1665.33^{b}	1753.21^{c}	<0.001
	s	250.78	191.95	251.52	152.78	192.93	
42nd	$\bar{x}$	1961.67^{a}	1985.97^{a}	1999.65^{ac}	2083.59^{bc}	2146.3^{b}	0.002
	s	289.95	214.49	291.48	185.28	229.17	

*Kruskal-Wallis test

$\bar{x}$ = mean; s = standard deviation; means within rows without common superscripts differ significantly $^{a,b,c}P<0.05$; K = control group; P1 = feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 = feed mixture + 0.5 g of propolis/kg of feed mixture; P3 = feed mixture + 1.0 g of propolis/kg of feed mixture; P4 = feed mixture + 20 g of bee pollen/kg of feed mixture.

K group. The P1, P3, and P4 groups had a 2.5% mortality rate, whereas in the P2 group no deaths of experimental animals were recorded. The study has shown that there was a statistically significant difference in mortality in the K group (10.0%; 4/40) in comparison to all the other experimental groups (1.9%; 3/160) (Fisher's exact test; p=0.031). This result is consistent with the result of the study done by Khojasteh Shalmany and Shivazad (18), who found that the chickens fed with the addition of propolis to the amounts of 200 and 250 mg/kg of feed mixture had much lower mortality rate than the K group of chickens. This study is also consistent with the results of the study by Omar et al. (19), who found that the chickens fed with the addition of propolis had better survival rate (95%) than the K group of chickens (88.3%). The positive effect of propolis/bee pollen in terms of mortality of chickens in this and similar studies can be explained by powerful antimicrobial (bactericidal, antiviral, antifungal and antiprotozoal) properties of bee products. Due to the mentioned antimicrobial properties, bee products are considered to prevent subclinical infections in chickens, while simultaneously acting as growth promoters (19). Apart from their antimicrobial properties, propolis/bee pollen have positive effects on the mortality of chickens as a result of their stimulation of the immune system by boosting the immune response of chickens and enhancing their resistance, thus directly reducing mortality in comparison to the K group chickens (18,19).

Table 3: Weekly weight gains of chickens (g)

Weeks	Statistical parameters	Group of chickens					*P – value
		K	P1	P2	P3	P4	
1st	$\overline{x}$	84.74[a]	88.54[ab]	90.65[ab]	94.26[b]	100.63[c]	<0.001
	s	17.20	16.01	13.26	13.66	17.55	
2nd	$\overline{x}$	177.19[a]	177.33[a]	192.55[ab]	205.64[b]	210.41[b]	0.002
	s	55.68	45.83	50.26	40.55	38.77	
3rd	$\overline{x}$	304.53[a]	348.38[b]	345.83[b]	378.67[bc]	391.10[c]	<0.001
	s	106.41	97.72	93.27	88.04	82.74	
4th	$\overline{x}$	410.36[a]	421.59[ab]	435.98[b]	420.62[a]	443.67[b]	0.029
	s	66.18	57.09	75.02	36.25	52.10	
5th	$\overline{x}$	508.00[a]	504.05[a]	493.65[a]	524.82[a]	566.08[b]	0.009
	s	87.17	83.40	117.53	68.36	94.89	
6th	$\overline{x}$	435.64	404.82	399.70	418.26	393.10	0.123
	s	64.88	75.78	74.85	68.27	84.59	

*Kruskal-Wallis test

$\overline{x}$ = mean; s = standard deviation; means within rows without common superscripts differ significantly [a,b,c]P<0.05; K = control group; P1 = feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 = feed mixture + 0.5 g of propolis/kg of feed mixture; P3 = feed mixture + 1.0 g of propolis/kg of feed mixture; P4 = feed mixture + 20 g of bee pollen/kg of feed mixture.

The values of the investigated haematological parameters in chickens' blood on the 21st day of the fattening period according to the specific group of chickens are shown in Table 4. Statistical analysis has shown that there was a statistically significant difference in the values of MCV, MCHC and haematocrit, leukocyte count and relative Mo ratio, while there was no statistically significant difference in the erythrocyte count, values of haemoglobin and MCH and the relative ratio of He, Ly, Eo and Ba between the analysed groups of chickens on the 21st day of the fattening period. The results of this study are in contrast to those of the study by Eyng et al. (20) who found no statistically significant differences in the relative ratios of heterophils, lymphocytes, monocytes and basophils between the control and experimental groups. However, they did find statistically significant differences in the relative ratio of eosinophils between the groups. Furthermore, taking into consideration the values of the monocytes determined in our study, it is clear that they are opposite to those of the study by Eyng et al. (20). Specifically, the highest relative ratio of monocytes in our study was recorded in the K group of chickens and the lowest in the P4 and P3 groups of chickens (the highest amount of bee pollen in P4 and the highest amount of propolis in P3), in contrast to Eyng et al. (20) who found that the highest relative ratio of monocytes was determined in the blood of chickens that were fed with the addition of 200 ppm of propolis (mean amount of propolis), and the lowest relative ratio of monocytes was found in the blood of the K group of chickens.

The values of the investigated haematological parameters in chickens' blood on the 42nd day of the fattening period according to the specific group of chickens are shown in Table 5. Statistical analysis has shown that there was a statistically significant difference in the relative ratio of He, Ly and Mo, while there was no statistically significant

Table 4: Haematological parameters in chickens' blood on the 21st day of the fattening period

Parameters	Group of chickens $\overline{x}$ ± s					*P* – value
	K	P1	P2	P3	P4	
E (10^{12}/L)	2.29±0.13	2.21±0.14	2.32±0.19	2.29±0.13	2.38±0.15	0.186*
Hb (g/L)	107.70±6.31	105.10±5.47	110.90±8.36	107.90±5.36	111.40±7.62	0.122†
Htc (L/L)	0.274^{ac}±0.017	0.265^{a}±0.015	0.281^{abc}±0.022	0.285^{bc}±0.014	0.292^{b}±0.020	0.015†
MCV (fL)	119.46^{ab}±4.08	119.92^{a}±1.17	121.23^{ab}±2.26	124.38^{b}±2.93	122.86^{ab}±4.76	0.009*
MCH (pg)	47.01±1.67	47.56±1.14	47.77±1.02	47.15±1.27	46.87±1.40	0.515*
MCHC (g/L)	393.70^{a}±9.17	396.40^{a}±8.24	394.20^{a}±6.51	379.10^{b}±5.17	381.10^{b}±8.71	<0.001*
L (10^{9}/L)	13.80^{ab}±6.36	12.80^{ab}±5.51	12.40^{a}±2.95	19.20^{b}±5.35	13.40^{ab}±4.62	0.029*
He (%)	42.50±6.10	48.30±5.33	47.90±7.34	48.90±6.08	47.20±7.74	0.211*
Ly (%)	50.40±5.50	48.70±4.79	48.80±8.39	48.80±6.07	50.60±7.81	0.934*
Eo (%)	2.70±2.06	1.60±1.51	1.70±1.25	1.40±1.51	2.20±0.92	0.308*
Mo (%)	3.70^{a}±3.06	1.10^{b}±1.85	1.50^{abc}±1.27	0.70^{b}±0.95	0.10^{c}±0.32	<0.001†
Ba (%)	0.70±0.82	0.30±0.68	0.10±0.32	0.20±0.42	0.00±0.00	0.063†

*ANOVA; †Kruskal-Wallis test

$\overline{x}$ = mean; s = standard deviation; means within rows without common superscripts differ significantly $^{a,b,c}P<0.05$; K = control group; P1 = feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 = feed mixture + 0.5 g of propolis/kg of feed mixture; P3 = feed mixture + 1.0 g of propolis/kg of feed mixture; P4 = feed mixture + 20 g of bee pollen/kg of feed mixture.

Table 5: Haematological parameters in chickens' blood on the 42nd day of the fattening period

Parameters	Group of chickens $\overline{x}$ ± s					*P* – value
	K	P1	P2	P3	P4	
E (10^{12}/L)	2.23±0.10	2.26±0.15	2.45±0.31	2.36±0.31	2.46±0.14	0.083*
Hb (g/L)	100.40±3.78	101.00±6.57	109.30±13.43	104.90±12.79	109.30±7.80	0.110*
Htc (L/L)	0.268±0.164	0.274±0.023	0.289±0.036	0.281±0.037	0.289±0.022	0.376*
MCV (fL)	120.18±3.94	121.19±4.95	118.04±4.66	119.44±4.56	117.72±4.18	0.390*
MCH (pg)	44.99±1.26	44.64±0.92	44.63±0.89	44.52±1.12	44.44±1.53	0.861*
MCHC (g/L)	375.90±13.75	368.90±10.37	378.50±10.86	373.30±7.90	377.80±10.04	0.278*
L (10^9/L)	30.80±15.32	28.40±17.04	32.80±17.05	44.00±12.75	36.60±13.30	0.189*
He (%)	37.50^{a}±8.48	50.30^{bd}±5.54	45.40^{be}±7.52	42.80^{ae}±7.12	53.70^{cd}±7.23	<0.001*
Ly (%)	58.70^{a}±7.88	47.00^{bc}±4.90	52.50^{ac}±7.98	55.30^{a}±7.39	43.80^{b}±6.68	<0.001*
Eo (%)	2.50±1.51	1.20±1.23	1.60±0.84	1.50±0.85	2.00±1.70	0.350†
Mo (%)	0.60^{ab}±0.70	0.90^{a}±0.74	0.10^{b}±0.32	0.30^{ab}±0.48	0.20^{b}±0.42	0.027†
Ba (%)	0.70±0.95	0.60±0.70	0.40±0.70	0.10±0.32	0.30±0.48	0.280†

*ANOVA; †Kruskal-Wallis test

$\overline{x}$ = mean; s = standard deviation; means within rows without common superscripts differ significantly $^{a,b,c}P<0.05$; K = control group; P1= feed mixture + 0.25 g of propolis/kg of feed mixture + 20 g of bee pollen/kg of feed mixture; P2 = feed mixture + 0.5 g of propolis/kg of feed mixture; P3 = feed mixture + 1.0 g of propolis/kg of feed mixture; P4 = feed mixture + 20 g of bee pollen/kg of feed mixture.

difference in the erythrocyte and leukocyte count, values of haemoglobin, haematocrit, MCV, MCH and MCHC and the relative ratio of Eo and Ba between the analysed groups of chickens on the 42nd day of the fattening period. This study, similar to one done by Omar et al. (19), has shown that all the experimental groups of chickens (P1-P4) had more erythrocytes and higher values of haemoglobin in relation to the K group of chickens; however, the aforementioned differences had no statistical significance. Furthermore, this study has shown that P2, P3 and P4 groups of chickens had more leukocytes in relation to the K group but having no statistically significant difference. In terms of the relative ratios of the individual leukocyte types, this study has shown that all the experimental groups of chickens (P1-P4) had higher values of heterophils and lower values of lymphocytes, eosinophils and basophils in comparison to the K group of chickens. With respect to monocytes, the study has shown that the P2, P3 and P4 groups of chickens had lower relative ratios of this type of leukocytes in comparison to the K group, whereas the P1 group of chickens had higher relative ratio of monocytes in comparison to the K group. In their research, Ziaran et al. (21) found that the chickens fed with the addition of propolis had a significantly lower relative ratio of heterophils and a significantly higher relative ratio of lymphocytes on the 47th day of fattening in comparison to the chickens of the control group, while there was no statistically significant difference in the relative ratios of eosinophils and monocytes between the chickens of the control and experimental groups. These results are in contrast to the results of the previously mentioned research by Ziaran et al. (21).

The results of this study are also contrary to the results of the study by Shahryar et al. (5), who found that on the 42nd day of fattening there were no statistically significant differences in the erythrocyte and leukocyte count and the relative ratios of heterophils, lymphocytes, eosinophils, monocytes, and basophils between the experimental groups of chickens fed with the addition of various amounts of propolis and the chickens in the control group.

In their study, Attia et al. (1) found that the chickens in the experimental groups fed with the addition of propolis and/or bee pollen had on the 35th day of fattening significantly more erythrocytes, higher values of haemoglobin, lower values of MCV, roughly the same values of MCH, and higher values of MCHC compared to the control group of chickens. These results are opposite to the results of our study, in which no statistically significant differences either in the erythrocyte count or values of haemoglobin MCV, MCH, and MCHC have been found. When analysing RBC parameters in chickens' blood in relation to the reference values of those parameters according to Wakenell (17), it can be said that the values of haemoglobin, haematocrit MCV, MCH, and MCHC and the erythrocyte count observed in our study are generally consistent with the previously mentioned reference values.

When analysing the effect of propolis and bee pollen on the values of RBC parameters in the blood of chickens on the 21st and 42nd days of fattening, this study has indicated that propolis and/or bee pollen positively affected the red blood cell count of broiler chickens. Accordingly, on the 21st day of fattening, higher values of haemoglobin, haematocrit MCV, MCH, and MCHC and more erythrocytes were determined in the experimental groups of chickens when compared to the K group of chickens. On the last day of fattening (42nd day), higher values of haemoglobin, haematocrit and more erythrocytes were again found in the experimental groups of chickens when compared to the K group. The aforementioned increase in RBC parameters determined in this study can be explained by better nutrient utilization in chickens fed with a feed mixture containing propolis or bee pollen, thus resulting in the better health condition of the chickens in general (1). Furthermore, studies by other authors have demonstrated that propolis significantly improves the digestive utilization of iron and haemoglobin regeneration efficiency (22,23). As regard to the effect of bee pollen, studies have shown that adding it to feed increases haemoglobin levels in animals' blood, as well as serum iron levels, resulting in better red blood cell counts (24). Furthermore, Omar et al. (19) have established that higher values of haemoglobin, erythrocyte count, total proteins and individual protein fractions in the blood of the chickens fed with the addition of propolis may be the result of a direct effect of propolis on the anabolic processes in the context of protein synthesis in the hematopoietic tissue of chickens, thus protecting body proteins against degradation. Bearing in mind that flavonoids are the main bioactive components

of propolis and bee pollen, which, among other effects, have strong antioxidant properties, it is most likely that these antioxidant properties prevent lipid oxidation in muscle cells, as well as in erythrocytes. Consequently, a decrease in lipid oxidation contributes to the better stability of red blood cell membranes and lower susceptibility of erythrocytes to haemolysis, thus having a positive effect on the erythrocyte count in the blood of the chickens fed with the mentioned additives (25,26).

When comparing WBC count parameters with the reference values according to Wakenell (17), it can be said that the values of the leukocyte count and the relative ratios of heterophils, lymphocytes, eosinophils, monocytes and basophils observed in our study are generally consistent with the previously mentioned reference values.

When analysing the effect of propolis and bee pollen on the values of WBC parameters in the blood of chickens on the 21st and 42nd day of fattening, this study has shown that the mentioned additives affect white blood cell count of broiler chickens. Accordingly, on the 21st day of fattening, the P3 group had higher and P1, P2, and P4 groups lower values of leukocytes when compared to the K group of chickens. During that period, a higher relative ratio of heterophils and lower relative ratios of lymphocytes, eosinophils, monocytes, and basophils were found in all the experimental groups of chickens. On the last day of fattening (42nd day), the P2, P3, and P4 groups had higher values and the P1 group lower values of leukocytes in comparison to the K group of chickens. Furthermore, during that period higher relative ratio of heterophils and lower relative ratios of lymphocytes, eosinophils, monocytes, and basophils were found in all the experimental groups of chickens. In birds, heterophils are phagocytic cells, and their primary role is to protect animals from the attacks of different microorganisms, whereas the primary role of lymphocytes implies cell-mediated and humoral immune response (27).

Taking into consideration the results of this study in terms of white blood cells, it can be concluded that the addition of propolis and bee pollen increases the relative ratio of heterophils and decreases the relative ratio of lymphocytes in the blood. The explanation may lie in the fact that synergism of different flavonoids that bee products contain has an immunosuppressive effect on the lymphoproliferative response in chickens due to nitric oxide production from macrophages which are responsible for the inhibition of DNA synthesis in different cells (21), and various studies have shown that bioactive components of propolis and bee pollen stimulate macrophage activation (28). Finally, it can be concluded that propolis and bee pollen have strong immunomodulatory effects through macrophage activation but no effect on the lymphocyte proliferation, as evident in this study from the differential blood count of chickens with higher relative ratios of heterophils and lower relative ratios of lymphocytes. Additional explanation of the results of this study as regards to the relative ratios of heterophils and lymphocytes lies in the fact that the chickens fed with the addition of propolis and bee pollen were thus exposed to antioxidant properties of flavonoids, as the most important bioactive components of these additives, and studies have shown that antioxidants intensify the phagocytic activity of heterophils (29).

Conclusions

The present study has undoubtedly determined that the addition of propolis and/or bee pollen to feed mixtures has significant positive effects on the performance parameters of broilers. Given the fact that lower mortality was recorded in all the experimental groups when compared to the control group of chickens, it can be concluded that the general health condition of chickens is improved by adding propolis and/or bee pollen into their feed mixture. Propolis and bee pollen also positively affect the values of the red blood cell count, since significantly higher values of MCV and haematocrit were recorded in the experimental groups of chickens in comparison to the control group on the 21st day of fattening. The investigated additives also have a significant positive effect on the values of white blood cells. Specifically, experimental groups of chickens had, in relation to the control group, significantly lower values of the leukocyte count and of the relative ratio of monocytes on the 21st day of fattening. On the 42nd day of fattening, the experimental groups of chickens had, in relation to the control group, significantly higher values of the relative ratio of heterophils and significantly lower values of the relative ratios of lymphocytes and monocytes.

Finally, this study has shown that propolis and

bee pollen (either separately or in combination) have a significant positive impact on the performance, the mortality and the values of the selected haematological blood parameters in chickens. To maximize the efficacy of the investigated natural feeding additives in commercial chicken production, it is necessary to further evaluate the administration level of these substances in chicken feed.

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VPLIV CVETNEGA PRAHU IN PROPOLISA NA RAST, SMRTNOST IN NEKATERE HEMATOLOŠKE KAZALNIKE PRI PIŠČANCIH BROJLERJIH

I. Klarić, M. Domaćinović, V. Šerić, I. Miškulin, M. Pavić, K. Paradinović

Povzetek: Namen raziskave je bil ugotoviti, kako cvetni prah ali propolis posamezno ali v kombinaciji vplivata na prirast, smrtnost in nekatere hematološke kazalnike pri piščancih brojlerjih. V raziskavo je bilo vključenih 200 piščancev brojlerjev pasme ross308. Piščanci so bili razdeljeni naključno v pet poskusnih skupin, v katerih sta bila enakomerno zastopana oba spola. Kontrolna skupina piščancev je ves čas raziskave prejemala navadno hrano za brojlerje, medtem ko so 4 poskusne skupine prejemale hrano z dodatkom cvetnega prahu in sicer skupina 1 (P1) 0,25 g propolisa in 20 g cvetnega prahu/kg hrane, skupina 2 (P2) 0,5 g propolisa/kg hrane, skupina 3 (P3) 1 g propolisa/kg hrane in skupina 4 (P4) 20 g cvetnega prahu/kg hrane. Telesna teža piščancev je bila statistično značilno višja 1. ($p < 0,001$), 2. ($p < 0,001$) in 5. ($p < 0,01$) teden raziskave v vseh poskusnih skupinah v primerjavi s kontrolno skupino. Podobno je bil tudi povprečen dnevni prirast pri piščancih iz poskusnih skupin statistično značilno višji 1. ($p < 0,001$), 2. ($p < 0,01$), 3. ($p < 0,001$), 4. ($p < 0,05$) in 5. ($p < 0,01$) teden raziskave. Tudi smrtnost je bila statistično značilno nižja v vseh poskusnih skupinah v primerjavi s kontrolno skupino ($p < 0,05$). Piščanci iz poskusnih skupin so imeli 21. dan poskusa v povprečju višje vrednosti MCV ($p < 0,01$) in hematokrita ($p < 0,05$) ter nižje povprečno število levkocitov ($p < 0,05$) in relativno razmerje MO ($p < 0,001$). Na 42. dan poskusa smo ugotovili višje vrednosti razmerja med heterofilci ($p < 0,001$) ter nižje relativne vrednosti limfocitov ($p < 0,001$) in monocitov ($p < 0,05$) pri piščancih iz poskusnih skupin v primerjavi s piščanci iz kontrolne skupine. Rezultati raziskave kažejo, da dodajanje propolisa ali cvetnega prahu izboljša splošno zdravstveno stanje piščancev brojlerjev ter posledično izboljša njihov prirast ter zviša telesno maso, zato bi se ju lahko uporabljalo kot dodatka k hrani za piščance brojlerje.

Ključne besede: piščanci; prirast; zdravstveno stanje; propolis; cvetni prah

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Original Research Article

EFFECT OF BIRTH WEIGHT, WEANING WEIGHT AND PREWEANING WEIGHT GAIN ON FERTILITY OF HOLSTEIN HEIFERS UNDER HOT MEXICAN CONDITIONS

Erika López[1], Francisco G. Véliz[2], Evaristo Carrillo[3], Ángeles De Santiago[1], José E. García[1], Miguel Mellado[1]*

[1]Department of Animal Nutrition, Autonomous Agrarian University Antonio Narro, Saltillo, [2]Department of Veterinary Science, Autonomous Agrarian University Antonio Narro, Torreon, [3]Techological Institute of Torreon, Torreon, Mexico

*Corresponding author, E-mail: mmellbosq@yahoo.com

Abstract: Holstein calves (n= 767) from a commercial herd were used to determine the effect of birth weight (BW), weaning weight (WW) and preweaning average daily gain (ADG) on subsequent reproductive performance of heifers inseminated with sex-sorted semen in a hot environment (25° N; mean annual temperature 23.7° C). BW were divided into three groups: <36, 36-39 and >39 kg. WW were classified as <66, 66-74 and >74 kg. ADG was categorized as <450, 450-520 and >520 g. Of the heifers initially bred, 7.8% failed to conceive with ≥5 services. Services/pregnancy were higher ($p<0.01$) in heifers with low BW (2.42 ± 1.39) than heifers with medium (2.13 ± 1.38) and high BW (2.05 ± 1.34). The proportion of heifers conceiving to the first service was lowest (27.9%; $p<0.01$) in heifers with the lowest BW compared to heifers weighing 36-39 kg (36.3%) and >39 kg at birth (40.3%). However, neither BW, WW, nor ADG significantly affected ($p>0.10$) all-service conception rate. Categories for BW, WW and ADG did not influence abortion rate (3.5% for all pregnant heifers). It was concluded that the heavier the Holstein calves are at birth and weaning, the shorter the age at calving under the present conditions, but low birth and weaning weight did not hamper all-service conception rate.

Key words: heifers; fertility; growth rate; abortion rate; conception rate

Introduction

The ability of dairy heifers to present an adequate growth rate for an earlier attainment of puberty, present normal estrous cycles, conceive at about 360 kg, sustain gestation to term, calve without assistance close to two years, and start their first lactation without puerperal disorders are critical component for dairy operations (1,2,3). Age at first calving is a vital factor affecting subsequent fertility and productivity of heifers (4,5,6) because an earlier age at first calving reduces rearing costs due to decreased labor, feed and building costs. Also, body weight at birth and growth rate before puberty can affect the subsequent milk produced during the first lactation (7,8,9) and survival rate to second lactation (10). Variability in preweaning and postweaning growth rates can lead to a large spread in the age at which heifers conceive for the first time (1). Optimum growth rates in replacement dairy heifers are necessary to breed their replacement heifers by about 15 months of age so that they calve for the first time at 24 months. This is broadly considered as the minimum age at which heifers are sufficiently grown to give birth without difficulty and to present a good milk yield during their first lactation.

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In zones of high ambient temperatures, heat stress may lead to reduced growth rates because neonatal calves have not developed adequate thermoregulation, which may be major contributors to the reduced weight gain during the first two weeks of life (11). Additionally, calves born to cows exposed to heat stress during late gestation (dry period) not only have compromised passive immune transfer but this heat stress has carryover effects beyond weaning, affecting negatively survival and milk production during the first lactation (12). Other authors have observed that Holstein calf performance in summer months either in extreme (13) or moderate climate conditions (14) showed a marked decline in growth rate as compared with winter conditions, which indicates that calves do not seem to be able to dissipate accumulated heat when daily high temperatures exceed their thermoneutral zone for healthy dairy calves.

Few studies have investigated the effect of preweaning growth variables in Holstein calves on their subsequent fertility in commercial dairy herds in hot environments. Furthermore, in many dairy record-keeping systems, calve and heifer growth rates are not recorded, thus maiden heifer growth performance is often unknown. The dairy industry would benefit significantly from being able to identify young age animals with altered birth weight and preweaning growth rate which would increase the risk of failing to conceive. The objective of this study was to determine the effect of birth weight (BW), weaning weight (WW) and preweaning average daily gain (ADG) on the reproductive performance of Holstein-Friesian heifers in a hot environment.

Material and methods

Animals and management

The study began in January, 2015 and ended in December of the same year, with the enrollment of 767 newborn calves (born as singletons) on one large commercial dairy farms located in northeastern Mexico (26°N; 23.7°C mean annual temperature). Calves were sired by seven different bulls (artificial insemination, both sex-sorted and conventional semen) and these were equally distributed across this contemporary group. Calves were removed from the dam within 30 min of birth and before suckling could occur. Calves eligible for enrollment had to be singletons, weight ≥ 29 kg, and born unassisted or with minimal difficulty (easy pull). Calves were fed 3.8 L of colostrum using an esophageal tube feeder by about 2 h of age. The average calf in the study weighed 38.4 kg, so 3.8 L of colostrum was approximately 10% of BW. Immediately after the colostrum feeding, calves were moved into individual 3.8-m^2 (2.5 x 1.5 m) open roofed pen, without bedding and provided twice-daily feedings (at 0700 and 1700 h) of 2 L of a commercial milk replacer containing 20% crude fat and 22% CP, diluted to achieve 11% DM. Calves remained in these pens until weaning (7 to 8 wk of age). Calves were open bucket fed and had access to free-choice water and pelleted calf starter. Body weight was measured with a mechanic scale at birth (before colostrum ingestion) and at weaning. Average daily gain was determined by subtracting weight at weaning from birth weight and then dividing by age (d) of calves.

After weaning the heifers were housed in groups of 30 animals per pen. The open-lot dirt-floor pens were located in a naturally ventilated area with plenty of shed structures. Feed bunks were located along the front of each pen allowing for 0.50 m of open bunk space per animal. Heifers were fed a TMR twice daily formulated to meet or exceed the nutritional requirements of Holstein heifers weighing 250 kg and gaining 0.8 kg/d (15). Diets contained soybean meal and ground shelled corn as the base ingredients of concentrate mix; the forage portion of the diet was oat hay. Heifers were fed ad libitum with an approximate refusal of 10% of that offered. Orts were cleaned out of the feed bunks each day, with new feed delivered twice daily. Water was available ad libitum through a water bowl in each pen. Heifers were also given ad libitum access to a trace mineral supplement.

Reproductive management

All heifers were vaccinated against diseases that impair reproduction functions, such as brucellosis (Brucella abortus RB51®, Intervet Mexico, Huixquilucan, Mexico), infectious bovine rhinotracheitis, bovine viral diarrhea, bovine respiratory syncytial virus, para-influenza and leptospirosis (5-varieties; Gold FP5®, Zoetis, Mexico D.F., Mexico).

Heifers were submitted for AI when detected in estrus after visual observation twice daily. Artificial

insemination (AI) was conducted following the standard a.m./p.m. rule. Commercial sex-sorted frozen-thawed semen from 5 different bulls from the USA was used across all months of the year. The straws contained 2 million spermatozoa and the sperm cells were deposited in the uterine body. Bulls were almost used equally across heifers included in the study, therefore, bulls were fairly evenly distributed among the contemporary heifers.

Pregnancy was detected by rectal palpation of the uterus by the herd veterinarian (same veterinarian throughout the study period) about 45 days post-AI. First-service conception rate was defined as the percentage of heifers that conceived at the time of their first breeding. All-service conception rate was defined as the number of heifers that conceived out of the ones that were inseminated ≤5 services. Days to first service was defined as the time (d) from when a heifer was born until the first time she was bred. Interval to pregnancy was the number of days from calving to AI resulting in pregnancy among the heifers inseminated. Heifers not pregnant with ≤5 services days was calculated as the number of heifers which did not get pregnant with a maximum of 5 services, divided by the number of heifers to be bred (expressed as a percentage).

Statistical analysis

The GENMOD procedure of SAS (SAS Inst., Inc., Cary, NC) was implemented to assess the effect of BW categories, weaning weight classes and preweaning average daily gain on first-service conception rate, all-service conception rate, abortion rate and calving rate. The procedure used a generalized linear model based on the logit transformation and binominal distribution of data. For continuous variables (interval to first AI, birth to conception interval among pregnant heifers and age at first calving), general linear models (GLM of SAS) procedure was used. Month of birth was included in the model as a covariate. The differences between individual groups were compared by the PDIFF option after least square means (LS means). After limiting the number of services per conception to heifers with a confirmed pregnancy diagnosis, the effect of growth traits on the number of services per conception was evaluated by the bivariate Wilcoxon rank sum test (non-parametric; proc npar1way; SAS). This procedure was used because the UNIVARIATE option of SAS indicated that services per pregnancy did not follow a normal distribution. The CORR procedure of SAS was used to determine the correlation coefficients between the growth traits. Statistical significance was defined as $P<0.05$.

Results

The reproductive performance of heifers according to their BW is presented in Table 1. The time to the first insemination was ten days shorter ($p<0.01$) for heifers with heavier BW than heifers with medium and low BW. No difference for this variable was observed between heifers whose BW was between 36 and 39 kg and those weighing <36 kg at calving.

As expected, the age at conception was much shorter ($p<0.01$) in heifers with the heaviest BW than heifers that were lighter at calving. BW significantly affected first-service conception rate with the heaviest heifers at parturition having the highest first-service conception rates and the heifers with light birth weights presenting the lowest first-service conception rates (Table 1). However, all-service conception rate did not differ ($p>0.10$) among groups regarding BW. Abortion rate was higher ($p<0.01$) for heifers with the medium BW, with no differences between the heaviest and lightest heifers at birth. BW had no significant effect on percentage of heifers failing to conceive with a maximum of five services.

Heifer fertility in relation to their WW is presented in Table 2. The average age to first breeding was shorter ($p<0.01$) in those heifers with the highest WW compared with heifers with low WW. Likewise, heifers with the heaviest WW presented shorter ($p<0.05$) days to conception and at calving than heifers with lighter weight at weaning. Conception rate at first service of the heaviest heifers at weaning differed ($p<0.01$) by as much as 13 percentage points compared with heifers with the lightest WW. Both all-service conception rate and calving rate did not differ among groups of heifers regarding WW.

The reproductive performance of heifers according to their preweaning ADG is presented in Table 3. Both groups of heifers with medium and high preweaning growth rate were younger ($p<0.01$) at first breeding than heifers with the

Table 1: Nulliparous Holstein heifer fertility in relation to their birth weight

Item	Birth weight (kg)		
	<36 (n=233)	36-39 (n= 197)	>39 (n=337)
Age at first breeding (days)	400 ± 36[a]	400 ± 41[a]	390 ± 26[b]
Age at conception (days)	434 ± 46[a]	424 ± 42[b]	419 ± 36[b]
Age at calving (months)	23.9 ± 1.6[a]	23.5 ± 1.4[b]	23.3 ± 1.2[b]
Services/pregnancy (pregnant heifers)	2.42 ± 1.39[a]	2.13 ± 1.38[b]	2.05 ± 1.34[b]
First-service conception rate (%)	27.9 (65/233)[a]	36.3 (57/157)[b]	40.3 (152/377)[b]
All-service conception rate (%)	90.1 (210/233)	94.9 (149/157)	92.6 (349/377)
Abortion rate (%)	2.4 (5/210)[a]	6.7 (10/149)[b]	2.9 (10/349)[a]
Failure to conceive with 5 services (%)	9.9 (23/233)	5.1 (8/157)	7.4 (28/377)
Calving rate (%)	88.0 (205/233)	88.9 (139/157)	89.9 (339/377)

[a,b]Values followed by the same superscript do not differ ($p<0.01$).

Table 2: Nulliparous Holstein heifer fertility in relation to their weaning weight

Item	Weaning weight (kg)		
	<66 (n=282)	65-74 (n= 228)	>74 (n=257)
Age at first breeding (days)	399 ± 34[a]	395 ± 33[ab]	391 ± 31[b]
Age at conception (days)	428 ± 37[a]	428 ± 46[a]	418 ± 39[b]
Age at calving (months)	23.7 ± 1.3[a]	23.6 ± 1.6[a]	23.3 ± 1.3[b]
Services/pregnancy (pregnant heifers)	2.28 ± 1.28	2.18 ± 1.37	2.02 ± 1.30
First-service conception rate (%)	29.1 (82/282)[a]	36.8 (84/228)[b]	42.0 (108/257)[b]
All-service conception rate (%)	89.7 (253/282)	93.0 (212/228)	94.2 (242/257)
Abortion rate (%)	2.8 (7/253)	4.7 (10/212)	3.3 (8/242)
Failure to conceive with 5 services (%)	9.9 (28/282)	7.0 (16/228)	5.8 (15/257)
Calving rate (%)	87.6 (247/282)	88.6 (202/228)	91.1 (234/257)

a,bValues followed by the same superscript do not differ ($p<0.01$).

Table 3: Nulliparous Holstein heifer fertility in relation to their average preweaning daily weight gain

Item	Average daily gain (g)		
	<430 (n=223)	430 - 520 (n= 222)	>520 (n=322)
Age at first breeding (days)	401 ± 33[a]	393 ± 30[b]	393 ± 35[b]
Age at conception (days)	427 ± 37	424 ± 38	423 ± 45
Age at calving (months)	23.6 ± 1.3	23.5 ± 1.3	23.5 ± 1.5
Services/pregnancy (pregnant heifers)	2.19 ± 1.22	2.27 ± 1.44	2.10 ± 1.30
First-service conception rate (%)	31.8 (71/223)	37.8 (84/222)	37.0 (119/322)
All-service conception rate (%)	89.7 (200/223)	92.8 (206/222)	93.5 (301/322)
Abortion rate (%)	3.5 (7/200)	3.4 (7/206)	3.7 (11/301)
Failure to conceive with 5 services (%)	10.3 (23/223)	7.2 (16/222)	6.5 (21/322)
Calving rate (%)	86.6 (193/223)	89.6 (199/222)	90.1 (290/322)

a,bValues followed by the same superscript do not differ ($p<0.01$).

lowest growth rate before weaning. Preweaning ADG did not affect all other reproductive variables.

Discussion

The calf population exhibited a 1.8-fold natural variation in birth weight (29-51 kg). This large variability in birth weight reflects ample growth rates during prenatal development. In the present study, it is believed that environmental factors were more important in influencing low BW than genetic factors. Chronic exposure in utero to elevated environmental temperatures, as the ones experienced by pregnant cows in the present study, greatly reduce birth weight of calves (11,12) and low BW calves fail to attain the same weight at 12 mo of age achieved in calves from dams not experience heat stress when dry (16).

Surviving calves with birth weight <36 kg showed suboptimal values for most reproductive variables compared with heavier calves at birth. For instance, lighter calves at birth required more days to first breeding and conception, which led to a greater age to first calving. For this last trait, it has been also reported that heifers with high birth weight were younger at first calving (17). Calves that were born small did not exhibit early catch-up growth to weaning at eight weeks of age (r= 0.16 for BW and ADG); therefore, it seems that lighter calves at birth resulted in delayed puberty in these animals. An association between body weight gain and timing of puberty has been reported in heifers (18,19). Also, low BW calves presented lower first-service conception rate and greater services per conception than heavier calves at birth.

Overall first-service conception rate was 36%, which is close to the 39% achieved in heifers bred with sexed semen in intensive systems in the United States (20). All-services conception rate in heifers with low BW was similar than that observed in medium and high BW heifers. This result is in line with observations of other researchers (21,22) who reported that lighter heifer calves at birth continued to have lesser BW during their first lactation, but this did not affect subsequent fertility. Thus, lower calf BW in this study was significantly associated with future all-services pregnancy rate but was negatively associated with other important reproductive variables such as the interval from birth to first calving. Age at first calving is an important factor in determining the length of the nonproductive period as well as affecting subsequent fertility (1,5). The decision on when to breed heifers is a management one, but it is influenced by pre-and post-weaning growth rate of heifers (23). The fertility of the heifers at this point will then affect age at first calving on any particular dairy farm.

Heifers whose birth weight was very low (<36 kg) calved at 23.8 months which is within the range of 23 to 24.5 mo considered to maximize dairy farm profitability (1,24). These data do not confirm previous reports of delays in age to first calving in dairy cattle in other temperate countries (5,6,25). These data suggest that even with very low BW heifers in the present study achieved the first calving at about 24 mo, which suggests that calves with low birth weight can subsequently show accelerated growth when feeding conditions improve, apparently compensating for the initial setback. Research suggests that, although compensatory growth can bring rapid benefits, it is also associated with a variety of costs that are not evident until later in adult life. These data also show that heifers with poor initial conception rates or with higher services per conception clearly calved later than those which conceive earlier in life, but, at the end, these heifers attained adequate pregnancy rates and did not present a calving delay for maiden dairy heifers.

The intervals from birth to first breeding and birth to conception was longer in heifers with low WW compared with heavier heifers at weaning. It is possible that lighter heifers at weaning reached puberty later than animals with higher WW. It has been observed that prepubertal growth rate is positively associated with the proportion of heifers cycling at the breeding age (26). This situation would increase cases of first ovulations not accompanied by overt estrus signs and the occurrence of a short luteal phase, with the subsequent fall in progesterone helping to promote the first behavioral changes (27). On the other hand, probably puberty was reached earlier in the heaviest heifers at weaning as a result of the inverse relationship between growth rate and age at puberty (28,29).

First-service conception rate was higher in heifers with high WW compared with light heifers at weaning. Probably the heaviest heifers at weaning presented more mature reproductive tracts as a result of alternating peaks of oestradiol and progesterone post-pubertally. This possibly

improved fertility as evidence shows pregnancy rates of 57% and 78% when beef heifers were served at the first compared with the third observed oestrus (30). On the other hand, heifers with lighter WW probably were served with fewer estrous cycles previous to their first insemination which resulted in lower conception rate at first service and greater services per conception. Similar to other studies where increased WW has been associated with lower age at calving (31), in the present study heavier heifers at weaning had a shorter birth to calving interval.

Preweaning ADG ranged from 175 to 912 g. All heifers were managed in the same way, so growth rate did not relate to plane of nutrition provided. This excessive variation was probably linked with heat stress or disease (32,33). The only reproductive variable affected by preweaning ADG was age at first service with a longer interval from birth to first insemination in heifers with the lowest preweaning ADG. Possibly the low growth rate of calves in this study prevailed during the prepubertal period, which could have delayed the occurrence of estrus. Heavier heifers, on the other hand, probably attained puberty earlier as time to puberty is negatively associated with prepubertal level of feeding (26). Additionally, heifers with greater prepubertal ADG present high amplitude LH pulses and high-frequency low amplitude LH pulses at a younger age (34). Other authors have observed that heifers with faster growth rates and higher concentrations of insulin-like growth factor-I (IGF-I) and glucose, breed earlier (2).

Preweaning ADG did not affect all-services pregnancy rate, which is in line with findings of other researchers who have found that post-natal growth rate does not influence future reproductive success in sheep (35) and dairy cattle (6).

Under the conditions prevailing in this experiment, which ensured maximum nutrient intake from birth to gestation, preweaning daily weight gain did not impair most of the measured reproductive functions. Thus, our results suggest that the type of preweaning growth in well-fed calves is not an important consideration for future reproductive performance of Holstein heifers.

In conclusion, a significant finding of the current study was that light birth and weaning weights had an impact on some subsequent fertility traits in maiden heifers. However, low birth weights did not hamper important reproductive traits such as all-service conception rate. Additionally, low birth and weaning weight were not an obstacle to begging artificial insemination breeding by <13.5 months, which resulted in calvings close to the recommended 24 months of age. Thus, despite the fact that birth and weaning weights alters some reproductive performance of Holstein heifers in a hot environment, this should not be considered when selecting replacement animals as catch up' feeding before puberty allow these heifers to achieve target live weight to calve close to 24 months.

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POVEZAVA MED TELESNO MASO, MASO OB ODSTAVITVI IN PRIRASTOM V ČASU PRED ODSTAVITVIJO NA PLODNOST TELIC HOLŠTAJN-FRIZIJSKE PASME V VROČIH KLIMATSKIH RAZMERAH MEHIKE

E. López, F. G. Véliz, E. Carrillo, Á. De Santiago, J. E. García, M. Mellado

Povzetek: Pri telicah holštajnske pasme (n = 767) iz komercialne črede smo ugotavljali vpliv telesne mase ob rojstvu, telesne mase ob odstavitvi ter dnevnega prirasta od rojstva do odstavitve na kasnejše reproduktivne sposobnosti telic, ki smo jih osemenili s semenom, ločenim glede na spol. Raziskava je potekala v vročih klimatskih razmerah (25 °C; povprečna letna temperatura 23.7 °C). Glede na telesno maso ob rojstvu smo telice razdelili v tri skupine: telice z maso, nižjo od 36 kg, telice z maso med 36 in 39 kg ter telice z maso več kot 39 kg. Glede na telesno maso ob odstavitvi pa smo razdelili telice v skupine z maso nižjo od 66 kg, maso od 66 do 74 kg ter maso, višjo od 74 kg. Enako smo živali razdelili v tri skupine glede na povprečni dnevni prirast in sicer na tiste, ki so v povprečju priraščale manj kot 450 g na dan, na tiste, ki so pridobivale od 450 do 520 g na dan, in na tiste, ki so imele povprečni prirast višji od 520 g na dan. Med vsemi telicami se jih 7,8 % ni zabrejilo tudi po peti osemenitvi. Razmerje med osemenitvami in zabrejitvijo je bilo višje pri telicah z nizko porodno težo kot pri drugih dveh skupinah ($p < 0,01$), in sicer je bilo pri telicah z nizko porodno težo potrebno povprečno 2,42 + 1,39 osemenitev do zabrejitve, pri skupinah s srednjo porodno težo je bilo potrebno 2,13 + 1,38 osemenitev, pri telicah z višjo porodno težo pa le 2,05 + 1,34 osemenitev do zabrejitve. Delež telic, ki so se zabrejile ob prvi osemenitvi, je bil prav tako najnižji v skupini z nizko porodno maso, le 27,9 %, medtem ko je bil v skupini s srednjo porodno maso 36,3, pri telicah z visoko porodno maso pa 40,3 5 ($p < 0,01$). Končno število brejih telic se glede na število osemenitev ni razlikovalo med skupinami ne glede na porodno maso, maso ob odstavitvi ali povprečni dnevni prirast. Prav tako telesna masa ni vplivala na pogostnost zvrgov, ki je bila 3,5 % pri vseh skupinah. Iz raziskave lahko ugotovimo, da se telice, ki so ob rojstvu težje, hitreje zabrejijo v toplih klimatskih razmerah, vendar pa telesna masa ob rojstvu ali ob odstavitvi ni povezana s povečanim pojavljanjem neplodnosti.

Ključne besede: telice; plodnost; prirast; pojavnost zvrgov; stopnja zabrejitve

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Original Research Article

MODULATION OF SYSTEMIC INNATE AND ADAPTIVE IMMUNE PARAMETERS IN WEANED PIGS BY SINGLE ORAL APPLICATION OF IMMUNOBIOTICS

Hrvoje Valpotić[1], Renata Barić-Rafaj[1], Ivona Žura Žaja[1], Silvijo Vince[1], Marko Samardžija[1]*, Romana Turk[1], Dražen Đuričić[2], Tomislav Dobranić[1], Przemyslaw Sobiech[4], Luka Cvetnić[3], Ivica Valpotić[1]

[1]Veterinary Faculty, University of Zagreb, Heinzelova 55, 10000 Zagreb, [2]Veterinary Practice Đurđevac, 48350 Đurđevac, [3]Croatian Veterinary Institute, Zagreb, Savska cesta 143, Zagreb, Croatia, [4]Faculty of Veterinary Medicine, Oczapowskiego 14, PL-10-719 Olsztyn, Poland

*Corresponding author, E-mail: smarko@vef.hr

Abstract: The aims of this study were to evaluate the effects of peroral treatment of 4-week old pigs at weaning (Day 0) with a single dose of levamisole (LEVA) or polyoxyethyle-polyoxypropylene (POE-POP), well known immunobiotics (IBC) and promising alternatives to dietary antibiotic growth promoters (AGP), on systemic innate and adaptive immunity by determining phagocytosis (PHC) and microbicidity (MBC) efficiency of their monocytes (MO) and granulocytes (GR), changes of serum C-reactive protein (CRP) and haptoglobin (HpG) profiles and kinetics of $CD4^+CD8^+$ T cells expression during 5 weeks following the treatments. Levels of CRP were decreased by either POE-POP or LEVA ($P<0.05$) at Day 7 and 21, respectively. LEVA-treated pigs had increased levels of HpG ($P<0.05$) at Day 14 and 21, whereas POE-POP-treated pigs had decreased and increased levels ($P<0.05$) at Day 7 and 21, respectively. Both IBC stimulated *in vitro* PHC of GR (from Day 7 to 35) and MBC of MO (at Day 35) from treated pigs ($P<0.05$). MO from POE-POP-treated pigs exhibited increased PHC ($P<0.05$) at Day 35, whereas GR from LEVA- or POE-POP-treated pigs showed increased MBC ($P<0.05$) at Day 7 and 35, respectively. The pigs treated by LEVA or POE-POP had higher proportions of $CD4^+CD8^+$ T cells ($P<0.05$) from Day 14 to 35 or Day 28 to 35, respectively. Tested IBC showed capability of stimulating particularly cellular components of nonspecific and specific immunity during early postweaning period before pigs reach adult immunocompetence values, and thus could be considered as potent immunostimulators in swine production.

Key words: synthetic immunobiotics; innate/adaptive immunity; weaned pig

Introduction

In swine production, at weaning pigs are immunologically immature and exposed to major stressful events, making them highly sensitive and susceptible to digestive disorders or enteric infections (1,2,3). At this time, the development of both innate and adaptive systemic and particularly local immunity (at gut mucosal surfaces) is critical in preventing the potential harmful effects of intestinal pathogens. Substantial research has been conducted over the past three decades to evaluate the potential antimicrobial agents for replacement of the in-feed antibiotics used as performance enhancers in swine production. This initial experience of an in-feed antibiotic growth promoters (AGP) ban in 1986 indicated that there was a reduced growth and increased morbidity/mortality in weaned pigs, which emphasized the therapeutic use of antibiotics in farms (4). The total ban of AGP in the EU since 2006 has had a serious impact on both performance and health status of weaned pigs underlining the need to develop alternative strategies (5). Since then, an intensive amount of research has been focused on

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the development of alternatives to AGP to maintain swine health and performance and many excellent reviews have been published on this subject (6,7,8). More recently, the focus has been directed to non-antibiotic approach offered by the use of immunobiotics (IBC), *i. e.* viable microorganisms (probiotics or microbiotics), their bioactive components (prebiotics), plant/fungi extracts (phytobiotics/fungibiotics), animal products or by-products (zoobiotics) and clay minerals (zeolites) as well as by a variety of other substances of natural (10,11,12,13) synthetic origin (14,15). Many of them have beneficial immunomodulatory properties and ability to prevent or reduce severity of systemic and local (intestinal) inflammatory disorders by stimulating non-specific innate and specific adaptive immunity in pigs.

Today, when the use of AGP is abandoned in animal production, there is intensive search for alternative strategies to control and prevent losses among weaned pigs, particularly due to enteric infections. These strategies include both natural (16,17,18,19,20) and synthetic IBC (21,22,23,24,25) preparations with antimicrobial detoxifying activities, as well as antioxidant, immunostimulatory and growth-promoting characteristics. However, the vast majority of these compounds produce inconsistent results and rarely equal AGP in their effectiveness. Therefore, it would appear that research is still needed in this area as the adequate alternative to AGP is not available as yet.

Considering our previous research on modulation of porcine immunity, health and growth benefits, the most promising results to date have been obtained with the synthetic IBC, levamisole (LEVA) and polyoxyethyle-polyoxypropylene (POE-POP) copolymers. The major positive effects of LEVA (23,24,25,26,27) and POE-POP (22,24) were to help weaned pigs to develop “appropriate”, but not over excessive active immune responses which may result in growth retardation in developing pigs during early postweaning period. They showed to act as the agents capable of stimulating components of the specific adaptive and nonspecific innate immunity, by increasing recruitment of either circulating $CD45^+$ lymphoid cells, $CD4^+$, $CD8^+$ T and $CD21^+$ B cells or intestinal $CD45RA^+$ naïve lymphoid cells, respectively, in immunologically immature early weaned pigs (24,25), and at the same time neither of them induced any detrimental effects on their haematological, serum biochemistry and gut histocytological homeostasis (29). Actually, this would be a logical continuation and essential supplementation of our aforementioned studies regarding the impact of LEVA and POE-POP simultaneously on immunity and on performance and health of weaned pigs which should be more largely documented as their immunomodulatory effects are also expected in these components of natural host defences.

Thus, the objectives of this study were to evaluate immunostimulatory effects of LEVA and POE-POP as potential alternatives to dietary AGP on components of porcine: (1) innate immunity such as blood phagocytic cells, such as monocytes (MO) and neutrophilic granulocytes (GR) and serum acute phase proteins (APP), such as C-reactive protein (CRP) and haptoglobin (HpG) profiles by establishing either their capability of phagocytosis (PHC) and microbicidity (MBC) or kinetics of their responses, respectively, and (2) adaptive immunity by determining changes in the proportion of extrathymic CD4CD8 double positive T lymphocytes in 28 days old weaned pigs during 35 days following the treatment with a single peroral dose of the IBC tested.

Materials and methods

Ethical and welfare approval

The experimental pigs were kept in facilities approved by the Croatian Association for Accreditation of Laboratory Animal Care, and in accordance with current regulations and standards issued by the Croatian Ministry of Agriculture. Experimental and management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC). The current study was supported by the grant no. 053-0532265-2255 from the Ministry of Science, Education and Sport of Croatia on January 2nd 2007.

Pigs

Sixty crossbred pigs (Topigs®) of both sexes (females and castrates) and body weight of 7.21±0.17 kg, progeny of six litters (from 3rd

parity sows) from a commercial swine farm in the eastern Croatia were used. 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 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).

Study design and treatments

The pigs were randomly divided into three groups comprising 20 animals each, ear-tagged with numbers 1-20 and kept in the separate pens (20 animals in each) of the same rearing facility under the same microclimatic conditions as previously described (25). After two days of accommodation at 28 days of age or Day 0 of the experiment, the pigs were perorally (*p.o.*) treated with a single dose of 10 mL as follows: (1) the controls received saline only, while the principals were treated with either (2) LEVA (Nilverm®, Pliva, Zagreb, Croatia) with 2.5 mg/kg body weight of the drug or (3) POE-POP (CytRx, Atlanta, GA, USA) with 2.5 mg/mL of the copolymer preparation as detailed earlier (24,25). The experiment was conducted throughout 35 days and was terminated at 63 days of age of the pigs, and during that period 7 pigs per group were sampled for peripheral blood at 7 day intervals starting at Day 0 before the treatments.

Blood sampling

The blood samples (10 mL) of 7 out of 20 pigs (ear-tagged with numbers 1-7 in order that same animals were sampled at each sampling day) from each group were taken ba *vv. cava cranialis* puncture using vacutainers (Beckton Dickinson, Plymouth, UK) and separated in three aliquots into either glass tubes (2 mL) with disodium EDTA (Sigma, St. Louis, MO, USA) as an anticoagulant (1 mg/mL) for flow cytometry analysis of extrathymic CD4CD8 double positive T cells or plastic heparinized tubes (2 mL) for the *in vitro* testing of MO/GR capability of PHC/MBC as well as into glass tubes (6 mL) without anticoagulant (Becton Dickinson, Rutherford, NJ, USA) for serum APP (CRP and HpG) determination.

Flow cytometry analysis

Peripheral blood lymphoid cells were isolated by Histopaque (specific density 1.077 g/mL; Sigma, Deisenhofen, Germany) density gradient centrifugation as detailed earlier (30). Murine monoclonal antibodies (mAbs) specific for porcine surface phenotypic markers CD4 (clone 74-12-4, isotype IgG2b) and CD8 (clone 76-2-11, isotype IgG2a) conjugated to either Pe/Cy5® or phycoerythrin (Abcam, Cambridge, UK), respectively, and a mAb to porcine CD45 (clone K252-1E4, isotype IgG1) conjugated to FITC (AbD Serotec, Kidlington, Oxford, UK) were utilized for flow cytometric (FCM) analysis to study identification/quantification patterns of respective peripheral blood lymphoid cell subsets. Briefly, a single cell suspensions (100 µL) were prepared in triplicates (comprising 10 000 cells each) and incubated with mAbs (50 µL) and processed as previously described (30). The fluorescence of the mAb-labelled porcine lymphoid cells was quantified using EPICS-XL Coulter flow cytometer (Coulter Electronics, Hialeah, FL, USA). The isotype-matched mouse immunoglobulins were used to detect a nonspecific fluorescence in the control cell suspensions as described previously (31). Only cells with a light scatter characteristics of lymphoid cells were analysed. More than 95% of such cells were $CD45^+$ for this lymphoid cell marker. The total proportion of T lymphocytes was calculated from the two-colour staining by the addition of $CD4^+ + CD8^+ + CD4^+CD8^+$ cells (32). The proportion of double positive $CD4^+CD8^+$ cells as a percentage of the total $CD4^+$ cell subset was calculated with the following formula: $(CD4^+CD8^+/(CD4^+CD8^+ + CD4^+))*100 = CD4^+CD8^+(\%)$ according to Zuckermann and Husmann (33).

Monocyte/granulocyte isolation and phagocytosis/microbicidity assays

Peripheral blood leukocytes (MO and GR) were isolated from heparinized venous blood using Ficoll-Hypaque 1077 (Sigma, St. Louis, MO, USA) density gradient centrifugation at 1500 x g for 30 minutes at 4 °C and their *in vitro* capabilities of PHC (cell ingestion) and MBC (cell digestion) were assessed as described earlier (34). Leukocyte rich supernatant was collected from plasma, washed trice in the medium 199 (MEM, minimal essential

medium, Institute of Immunology, Zagreb, Croatia) and concentration of obtained leukocytes was adjusted to 1 x 10^6/mL. The suspension of isolated cells was divided into aliquots of 0.25 mL, placed into small chambers (1.5 cm in diameter) and incubated at 37 °C with 5% CO_2 in the air for 30 min. Then supernatants were discarded and nonadherent cells were washed with MEM heated at 37 °C. The adherent cells *i. e.* phagocytes (MO and GR) remained in the chambers and 0.25 mL of suspension comprising 40 x 10^6/mL of viable cells (at least 99%) of yeast *Saccharomyces cerevisiae* was added to each chamber. The chambers were incubated for 30 min, washed and the cells were stained with 0.05% acridine orange (Sigma, St. Louis, USA) in MEM for 1 min. Then MEM was discarded and the chambers were covered by a cover slide and examined with fluorescence microscope at 800 x magnification. At least 100 of either GR or MO with phagocytosed yeast cells were counted. The obtained results were expressed as percentage of the cells that phagocytosed, where the % of PHC capability is presented as the number of ingested yeast cells/total number of yeast cells x 100, *i. e.* the ingestion index (ii), where the ii is the number of ingested yeast cells/ number of phagocytes. Capability of intracellular killing of yeast cells was determined based on differentiation between dead (stained red) and live (stained green) yeast cells, and was expressed as percentage were the % of MBC is the number of ingested dead yeast cells/total number of ingested yeast cells x 100, *i. e.* the digestion index (di).

Determination of serum acute phase proteins

Serum was separated from blood cells by centrifugation at 1200 g for 15 min, divided in two 0.5 mL aliquots and stored at -20 °C until analysed. The Tridelta Phase™ Range porcine CRP kit (Tridelta Development Ltd., Maynooth, County Kildare, Ireland) was used as a solid phase in sandwich immunoassay. The serum samples, including standards of known CRP content, were added to microtiter plates in order to bind to coated microwells. After washing to remove any unbound material, the horse radish peroxidase (HRP) labeled anti-porcine-CRP antibody was added to each well and incubated for 45 min. The microtiter plates were washed again to remove any unbound material and tetra methylbenzidine (TMB) substrate solution was added and the plates were incubated for 20 minutes at room temperature. The intensity of blue colour development changed to yellow by addition of a stop solution was measured by a microtiter plate reader BioTek Absorbance Microplate Reader EL x 808 (BioTek Instruments, Inc., Highland Park, VT, USA) and optical density was measured at 450 nm. The intensity of obtained absorbance for each well is proportional to the concentration of CRP in the tested serum sample. A standard curve, prepared from 7 standard dilutions in duplicates, was used for calculating the concentration of CRP in porcine serum. The serum concentration of porcine HpG was quantified spectrophotometrically using commercial reagent from Phase™ Range Haptoglobin Assay (Tridelta Development Ltd., Maynooth, County Kildare, Ireland) according to the manufacturer's instructions. Briefly, the HpG measurement is based on the fact that the peroxidase activity of free haemoglobin is inhibited at a low pH level (3 to 4). The haemoglobin binds to HpG in blood serum and at a low pH level it preserves the peroxidase activity of bound haemoglobin. Thus, the peroxidase activity of bound haemoglobin is directly proportional to the amount of HpG present in the serum sample. The absorbance of the samples was measured on an automated analyser Olympus AU 400 (Olympus diagnostica, Hamburg, Germany) at 600 nm. A calibration curve was prepared from five standard dilutions in duplicates in order to facilitate calculation of HpG concentration in porcine serum.

Statistical analysis

As the pigs were of a same breed, weighed approximately 7.20 kg at the weaning age of 26 days. Blood samples of the 7 pigs in each group were used as pooled sample. Data were analysed by Student's *t* test for independent samples using the StatisticaSixSigma software (StatSoft, Inc.). Each of two treated groups were compared with the control group of pigs without testing the time points within one group. Graphs were made using statistical Software SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with module PROC SGPLOT. The differences between obtained values for treated groups and the control group of pigs were considered significant at $P<0.05$ or lower.

Results

Performance and health status of pigs

Pigs treated with POE-POP and LEVA had a significant increase in live body weight, improved feed efficiency and average daily gain compared to the control pigs at the end of the trial. Both treatments positively influenced overall health status by reducing the incidence and severity of diarrhea and mortality which were documented in our previous studies (24,25).

Serum levels of CRP and HpG

The changes in APP profiles (CRP and HpG) in the serum of pigs treated with LEVA or POE-POP during 5 weeks following the treatment are presented in Figure 1 and Figure 2. None of IBC tested increased the concentration of serum CRP in treated pigs in comparison to the values obtained in the control pigs during the experimental period (Figure 1). However, the levels of CRP were significantly decreased in the pigs treated with either POE-POP or LEVA (P<0.05) at Day 7 and Day 21, respectively.

In the pigs that received LEVA significantly increased levels of serum HpG where recorded at Day 14 and Day 21 (P<0.05) of the experiment as compared to the respective values obtained in the control pigs (Figure 2). The values of HpG in the serum of POE-POP-treated pigs where either significantly decreased at Day 7 or increased at Day 21 (P<0.05, respectively).

Capability of phagocytosis and microbicidity of granulocytes and monocytes

Both IBC tested stimulated phagocytic activity of peripheral blood neutrophilic GR as demonstrated by the *in vitro* assays (Figure 3).

Thus, LEVA and POE-POP exhibited such stimulatory effects by significantly increasing phagocytic capability (ii) of GR from treated pigs at Days 7, 14, 28 and 35 as compared to those in the control pigs during the same period of the experiment. Also, the GR from pigs treated with either LEVA or POE-POP had significantly increased MBC at Day 7 and Day 35 (P<0.05), respectively (Figure 4).

The PHC of MO was significantly increased in the pigs treated with POE-POP (P<0.05) at Day 35 (Figure 5). However, the MBC of MO was significantly increased by both IBC (P<0.05) at Day 35 (Figure 6).

Proportion of CD4/CD8 double-positive T cells

The pigs treated with either LEVA or POE-POP had significantly higher proportions of peripheral blood $CD4^+CD8^+$ T lymphocyte than the control pigs from Day 14 to Day 35 and from Day 28 to Day 35 (P<0.05), respectively (Figure 7).

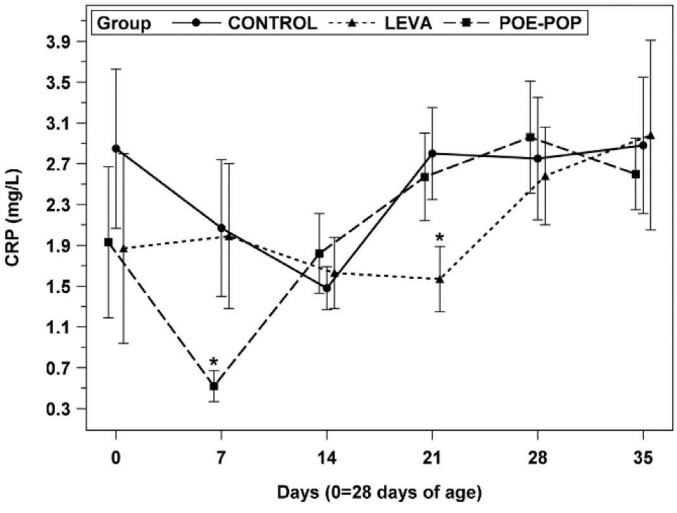

Figure 1: Concentration (mg/L) of C-reactive protein (CRP) in serum (Mean ± SEM) of weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at P<0.05 from the control values

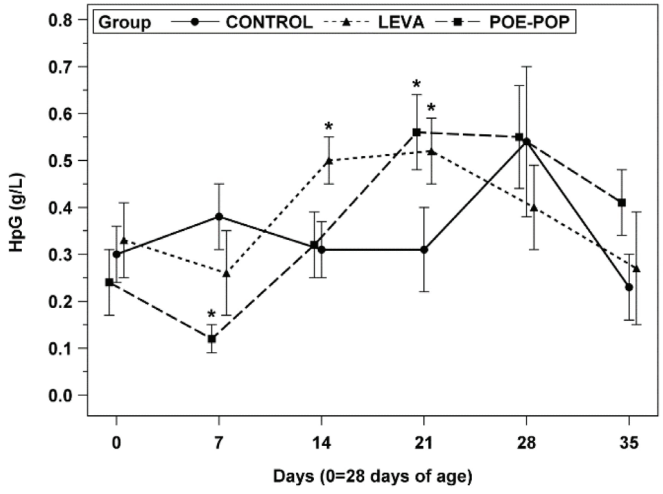

Figure 2: Concentration (g/L) of haptoglobin (HpG) in serum (Mean ± SEM) of weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

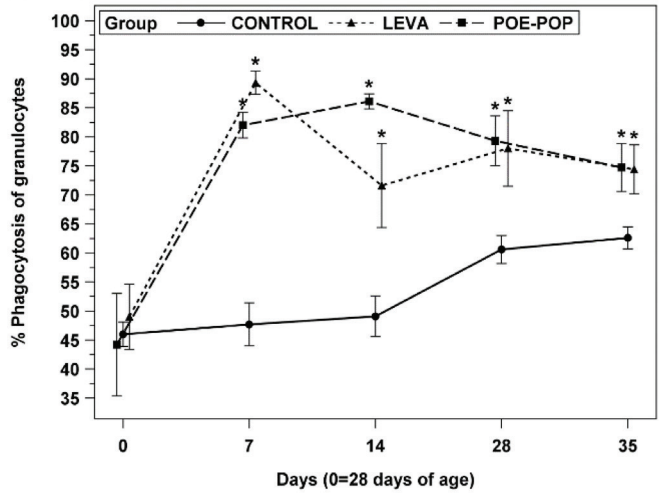

Figure 3: *In vitro* capability of phagocytosis (%) of peripheral blood neutrophilic granulocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (aLEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

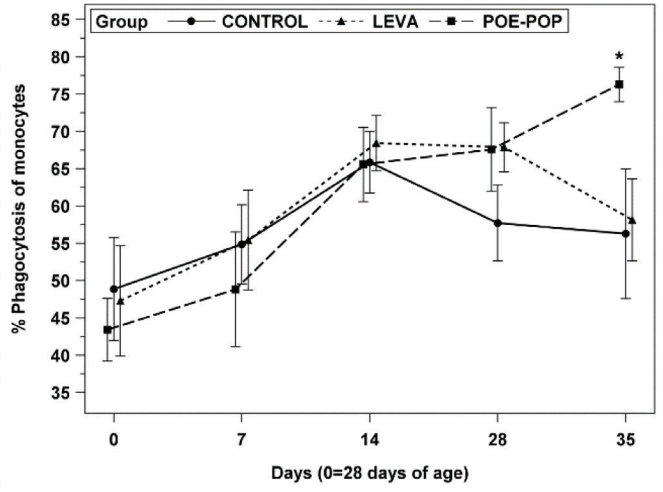

Figure 4: *In vitro* capability of microbicidity (%) of peripheral blood neutrophilic granulocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

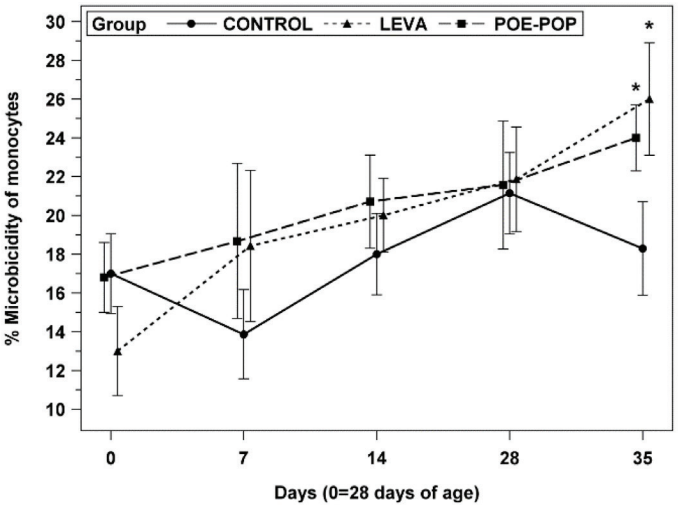

Figure 5: *In vitro* capability of phagocytosis (%) of peripheral blood monocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

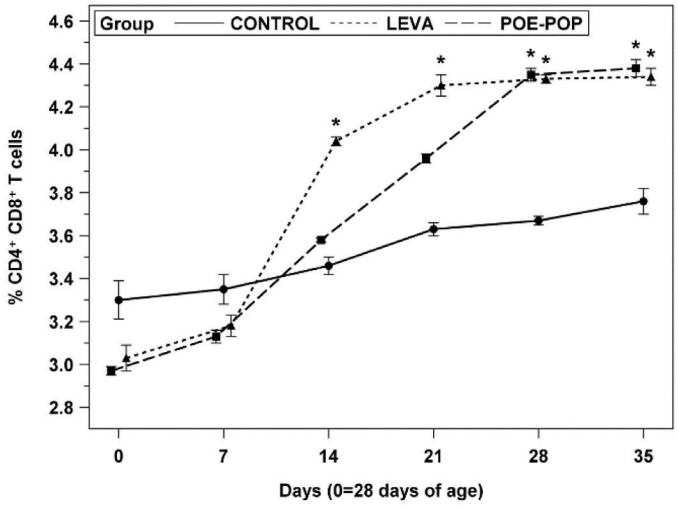

Figure 6: *In vitro* capability of microbicidity (%) of peripheral blood monocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

Discussion

In the present study, early responses of serum CRP and HpG in 4-weeks old weaned pigs induced by synthetic IBC either LEVA or POE-POP (at weaning or Day 0) were monitored (from Day 7 to Day 35 following the treatments) in order to obtain data on their potential modulating effects on porcine APP. Since there are no similar studies dealing with the effects of LEVA and/or POE-POP on porcine APP levels as far as we know, only comparison of obtained values for CRP and HpG with normal and acute ranges of these parameters in young pigs were logical and could be practical (35). Namely, the effects of IBC tested may influence normal (5-30 mg/L and 0.01-2.20 g/L) and acute (50-750 mg/mL and 3.00-8.00 g/L) ranges of either CRP or HpG levels, respectively, as important markers of diseases, health and welfare in pigs (36). Although, both IBC either decreased CRP or increased HpG serum levels, between Day 7 and Day 21 of the experiment, they did not affect normal ranges of their concentrations, which is a favourable outcome of our research. Particularly, in the cases of nonspecific immune responses to infective agents, inflammation, trauma and stress-induced conditions (37), exogenous manipulations with porcine innate immune system components such as CRP and HpG as performed with LEVA and POE-POP in the current study, may disturb rapid and significant changes in their serum concentrations and consequently abrogate their protective efficiency and value as diagnostic and prognostic indicators

of disturbed physiological homeostasis and health status of pigs (37). This is particularly true for weaned pigs during early postweaning period when they are exposed to a variety of pathogenic challenges (such as infectious diseases in field conditions), inflammatory lesion following natural injuries (such as tail and ear bites), acute inflammatory responses due to autoimmune or other disorders (such as arthritis and ulcerated umbilical hernia) and/or to stressors (38,39). Beside, these biological functions of CRP in not fully immunocompetent weaned pigs, its crucial role is in linking nonspecific innate immunity and specific adaptive immunity by interacting with specific receptors on phagocytic cells to mediate PHC or induce the release of anti-inflammatory cytokines (40).

Exogenous immunostimulation by IBC, such as LEVA and POE-POP firstly implies stimulation of non-specific innate immunity components, such as tissue macrophages, circulating MO and neutrophilic GR as well as NK-cells and γδ TCR^+ T lymphocytes (21). Accordingly, the current study investigated the impact of either LEVA or POE-POP as a potent IBC for porcine cellular components of specific adaptive immunity (23,25,24, respecitely), on PHC/MBC *in vitro* functions of blood MO and GR in weaned pigs during 5 weeks following the treatments at Day 0 or 4 weeks of age. The PHC function of GR have been stimulated by both IBC throughout 5 weeks following the treatments, whereas only the treatment with POE-POP increased *in vitro* phagocytosis of MO. Our findings are consistent with those reporting that the treatment with LEVA enhanced *in vitro* PHC function of neutrophilic GR, but not of MO in weaned pigs (41). However, since the experimental design of the latter study differ from that in the current study, *i. e.* LEVA was given intramuscularly in daily dose of 2.5 mg/kg of body weight for three consecutive days, this comparison should be taken advisedly. The *in vitro* MBC functions of GR and MO were enhanced equally by both IBC tested, although their stimulating effects were observed at Day 35 only. There are numerous studies similar to the current study performed on natural IBC such as probiotics, prebiotics and phytobiotics (11,12), in order to determine their modulating effects on either PHC or PBL functions of porcine circulating (16) and intestinal phagocytes (17,18,) but data obtained mostly remained inconsistent and inconclusive.

The results of the present study obtained on proportion of peripheral blood CD4/CD8 double positive T cells are consistent with previously reported finding that this subset of lymphocytes increases gradually with age (43). Namely, regardless the treatment applied all three groups of the pigs showed trend of increase in the proportion of this T cell subset with age during rather short period of 5 weeks of the experiment. However, both IBC tested stimulated significantly higher increase of CD4/CD8 double positive T cells from Day 14 to Day 35 (LEVA) and Day 28 to Day 35 (POE-POP) as compared to the values obtained in non-stimulated control pigs. This finding is also in agreement with earlier described functional characteristics of these cells as antigen primed T helper cells with memory/effector phenotype (44). Interestingly, the pigs stimulated with LEVA had increased proportions of circulating CD4/CD8 double positive T cells two weeks earlier than the pigs stimulated with POE-POP. Since both IBC tested were applied *p. o.*, it is very likely that they simultaneously reached the gut-associated lymphoid tissues (GALT) of the treated pigs. Such delay in the response to POE-POP as compared to that of LEVA could be due to differences in their immunogenicity for porcine intestinal CD4/CD8 double positive T cells. As established much earlier their predominant localization in the inductive sites of the GALT, such as Peyers patches, and their immediate migration into the circulation following antigen (or immunogen) stimulation (44) could be also a reason for that. Namely, our previous studies suggesting that LEVA exhibited more rapid and effective immunostimulating properties when used either as mucosal IBC (23,25) or adjuvant for mucosal vaccines (45) than POE-POP did (24,21,22, respectively). As we did not find similar data on modulating effectiveness of LEVA or POE-POP on porcine extrathymic CD4/CD8 double positive T cells we may only quote those found for the other circulating T cell subsets in weaned pigs. More recent studies have shown that POE-POP and, particularly LEVA stimulated almost the same kinetics of recruitment of $CD4^+$ and $CD8^+$ T cells in weaned pigs, *i. e.* from Day 21 to Day 35 (24) or from Day 14 to Day 35 (25), respectively.

Although, the IBC from natural sources will be more easily accepted by the consumers, both synthetic IBC tested are shown to be safe for pigs (29) and thus, for food safety they

must show to be effective in their purpose as potential immunostimulators (14,15), namely to act as alternatives to AGP and provide health and performance to pigs. Actually, LEVA and POE-POP showed capability of enhancing either functions or recruitment of cellular components of nonspecific/innate (MO/GR) and specific/adaptive immunity (extrathymic CD4/CD8 double positive T lymphocytes of memory/effector phenotype), respectively, and thereby help pigs to develop "optimal", not over excessive active immune responses during early postweaning period before they reach adult immunocompetence values. Immune activation can be counterproductive in healthy animals and divert nutrients from growth towards defence so measures must be taken to minimise the negative aspects to performance. Proper timing and duration of immunostimulation is essential but the fact that the increased immune response is sometimes associated with decreased performance is a problem that needs addressing. Therefore, it can be concluded that immunostimulatory effects of LEVA and POE-POP (without affecting normal/acute ranges of CRP and HpG serum levels as important markers of health status in pigs) provided a novel perspective for their use during early postweaning period before pigs reach adult immunocompetence values, and thus could be considered as potent immunostimulators in swine production.

Conflict of interest

Authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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VPLIV NA SISTEMSKO PRIROJENO IN PRIDOBLJENO IMUNOST PRI ODSTAVLJENIH PUJSKIH PO ENKRATNEM DODATKU IMUNOBIOTIKOV PREKO PREBAVNEGA TRAKTA

H. Valpotić, R. Barić-Rafaj, I. Žura Žaja, S. Vince, M. Samardžija, R. Turk, D. Đuričić, T. Dobranić, P. Sobiech, L. Cvetnić, I. Valpotić

Povzetek: Namen raziskave je bil ugotoviti vpliv lavamisola (LEVA) in polyoxyethylen-polyoxypropylena (POE-POP), znanih imunobiotikov, ki bi jih lahko uporabljali v prašičereji za boljši prirast namesto antibiotikov, na sistemsko prirojeno in pridobljeno imunost pri odstavljenih, 4 tedne starih pujskih. Vsem živalim smo LEVA in POE-POP dajali preko ust (peroralno) in tedensko v odobju petih tednov dodajanja imunobiotikov ugotavljali stopnjo fagocitoze in protimikrobno aktivnost monocitov in granulocitov, raven serumskega C-reaktivnega proteina (CRP), profil haptoglobina (HpG) ter raven prisotnosti limfocitov T, ki izražajo gena CD4 in CD8 v krvi. Rezultati so pokazali, da je bila raven serumskega CRP znižana pri pujskih, ki so prejemali tako LEVA kot POE-POP 7. in 21. dan raziskave ($p < 0,05$). Pujski, ki so prejemali LEVA, so imeli zvišan HpG 14. in 21. dan raziskave ($p < 0,05$), medtem ko so imeli pujski, ki so prejemali POE-POP, 7. dan raziskave HpG znižan, 21. dan pa zvišan ($p < 0,05$). Oba imunobiotika sta v pogojih in vitro dvignila stopnjo fagocitoze pri granulocitih in protimikrobno aktivnost monocitov na 35. dan raziskave ($p < 0,05$). Pujski, ki so prejemali en ali drugi imunobiotik, so imeli 35. dan raziskave višje vrednosti limfocitov T, ki so izražali CD4 in CD8, od vrednosti na 14. ali 28. dan raziskave ($p < 0,05$). Rezultati raziskave torej kažejo, da sta oba imunobiotika pozitivno vplivala na izbrane kazalce delovanja imunskega sistema in sta potencialno zanimiva za uporabo v prašičereji za spodbujanje delovanja imunskega sistema in s tem boljši prirast prašičev.

Ključne besede: odstavljeni pujski; umetni imunobiotiki; prirojena imunost; pridobljena imunost;

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