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HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF WEANED PIGLETS FED ON FODDER MIXTURE CONTAMINATED BY ZEARALENONE WITH ADDITION OF CLINOPTILOLITE

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The effect of zeolite clinoptilolite (CLIN) on some metabolic parameters in blood serum and haematological values in weaned piglets fed with increased levels of zearalenone (ZEN) was investigated over a 14-day period. The research involved four groups of weaned piglets aged 40 to 54 days. All groups were fed on fodder mixture for growing pigs containing 20% crude protein and 12.57 MJ ME/kg. The first control group (C1) received a concentrate mixture without added ZEN or mycotoxin adsorbent Min-a-Zel Plus®. The second control group (C2) was fed on fodder mixture containing 0.2% of modified clinoptilolite (Min-a-Zel Plus®; levels of zearalenone <5.1 ng/g). Piglets in the experimental group (E1) were fed on fodder mixture containing added zeralenone (3 mg/kg) (Sigma-Aldrich Co) and 0.2% of Min-a-Zel Plus® preparation. The second experimental group (E2) was fed on fodder mixture to which 3 mg/kg of zearalenone was added, but with no addition of Min-a-Zel Plus®. Oestrogenic effect of ZEN was evident in the sinergistic action with insulin which was manifested in an increased total protein level, lower level of glucose, triacylglycerols, serum iron, a higer level of cholesterol and higher aminotransaminase activity. The metabolic antioestrogen effect of ZEN was established in group E2, whereas group E1 manifested the agonistic effect due to oestrogen reduction by clinoptilolite.

Key words: clinoptilolite, zearalenone, biochemical parameters, haematological parameters, weaned piglets.

INTRODUCTION

The presence of mycotoxins has been established all over the world (Jurjević *at al*., 1999; Wood, 1992; Placinta *et al*., 1999) but in varying concentrations depending on climatic conditions, as well as on the conditions of cattle feed production. Research has shown that 25% of cereals in the world are contaminated with mycotoxins (Lawlor and Lynch, 2001). Zearalenon, produced

by fungi of the species *Fusarium*, is a non-steroid oestrogen which is often found in maize, oats, barley, and wheat grants, as well as in cattle feed mixtures (Bauer *et al*., 1980). The grains are contaminated in the field at an optimum temperature of 18-24 ^{o}C, and at a relative humidity of over 71%. In the organism biotransformation takes place in the liver and the digestive system and is the result of the activity of tissue enzymes and microflora (Galtier, 1999; Yiannikouris and Jouany, 2002). Zearalenon shows an affinity for oestrogen receptors (Powell-Jones *et al*., 1981; Mueller *et al*., 2004). Consequently, its effect is primarily linked to reproductive organs, although its influence on the hypothalamus and pituitary gland has also been established (Kitagawa *et al*., 1982). It is secreted through the gall bladder (65%), urine (21%), faeces (Gaumy *et al.*, 2001) and milk (Hagler *et al*., 1980). Following competitive binding to oestrogen receptors (ER) the synthesis of proteins is increased and proliferation of cells is induced, resulting in an increase of organ mass (anabolic effect). It is believed that its effect may not be restricted to oestrogen receptors alone (Murata *et al*., 2002), since it has been established that oestrogen, as well as phytoestrogens genestein and quercetin, are able to stimulate gene expression independent from classical ERs (Maggiolini *et al*., 2004). Acute toxicity was not found but lower concentrations can cause macroscopic changes in the ovaries (Gaumy *et al*., 2001; Zöllner *et al*., 2002; Yiannikouris and Jouany, 2002); swelling of pudenda in young gilts, changes in the external parts of sex organs of new-born and suckling pigs (Dacasto *et al*., 1995; Alexopoulos, 2001) and are the primary cause of rectum prolapse in pigs (Perfumo *et al*., 2002), and result in unfavourable effects on maturing oocytes and on embryo cultures in pigs (Alm *et al*., 2001). The development of early mastopathy in children is linked to the effect of zearalenone present in cereals (Szuets *et al*., 1997). A positive effect of the addition of organozeolite to the feed for piglets has been established (Stojić *et al*., 1998), sows and gilts (Kyriakis *et al*., 2002; Papaioannou *et al*., 2002), and diary cows (Enemark *et al*., 2003). Good adsorption abilities of aluminosilicate for ZEN *in vitro* have also been determined (Tomašević-Čanović Magdalena *et al*., 2003; Döll *et al*., 2004).

The aim of this study was to assess the influence of zearalenone on the general condition of organs and the organism as a whole, as well as on the metabolic status of pigs as the species most susceptible to its effects. Interest also focused on the possible reduction of its detrimental effect through the application of clinoptilolite, based on certain biochemical and haematological values and histological results.

MATERIALS AND METHODS

Animals and feeding

The research involved four groups of weaned piglets aged 40 to 54 days. All groups were fed on fodder mixture for growing pigs containing 20% crude protein and 12.57 MJ ME/kg. Each group comprised 5 female piglets bred from five sows and two boars. The first control group (C1) received a concentrate mixture without added ZEN, or mycotoxin adsorbent Min-a-Zel Plus®. The second control group

(C2) was fed on fodder mixture containing 0.2% of modified clinoptilolite (Min-a-Zel Plus®; levels of zearalenone <5.1 ng/g). Piglets in the experimental group (E1) were fed on fodder mixture containing an addition of 3 mg/kg concentration of zearalenone (Sigma-Aldrich Co) and 0.2% of Min-a-Zel Plus® preparation. The second experimental group (E2) was fed on fodder mixture to which 3 mg/kg of zearalenone was added, but with no addition of Min-a-Zel Plus®. Composition of Min-a-Zel Plus® is shown in Table 1; cation exchange capacity was 160 +/- 10 meq/100 g (Tomašević-Čanović *et al*., 2000).

Table 1 Chemical composition of the Min-a-Zel Plus®, organic modification of the zeolitic mineral-clinoptilolite with a long chain quaternary amonium salt

Compound	wt. (%)
SiO_2	63-68
Al_2O_3	11-14
Fe_2O_3	0.8-2.5
MnO	0.01-0.03
CaO	2.5-4.5
MgO	0.8-1.5
Na_2O	0.8-1.5
K_2O	1.0-2.0
L.I.	10.5-14.5

Blood samples, biochemical indicators and blood count

Blood samples were taken from the animals on the 8th and 14th day in order to determine haematological values and biochemical indicators. Five millilitre samples for hematological tests were obtained from the *v. Cavae cranialis* using a Venoject® vacutainer into a test tube containing an anticoagulant (EDTA), and 5 ml for biochemical tests. The levels of metabolites (glucose, urea, creatinine, cholesterol, bilirubin, total proteins, albumins, triglycerides), enzyme activities (aspartate amino transferase-AST, alanine aminotransferase-ALT, alkaline phosphatase-ALP, gamaglutamyltransferase-GGT, cholinesterase-CHE**,** creatine kinase-CK, lactate dehydrogenase-LDH and amylase), and mineral levels (iron-Fe) were established using the automatic analyzer Olimpus AU 640.

The number of erythrocytes, leukocytes, trombocytes, levels of haemoglobin and haematocrit were established using the Symex SF-3000 automatic counter. Blood smears were prepared and stained according to Pappenheim and investigated under a microscope in order to obtain at the differential blood count (Band Neutrophils, Neutrophils, Lymphocytes, Monocytes). The relative ratio of individual cells of leukocytes is given in percentages in relation to their total number.

Pathohistological tests

Upon the completion of the experiment the animals were euthanized by an intracardial injection of 0.3 ml/kg bw of T61$^{(R)}$ preparation (Intervet International B. V., Netherlands) and organ samples were taken (ovaries, kidneys, liver, spleen) for pathohistological investigations. Samples were fixed in a 4% solution of paraformaldehyde in a phosphate buffer at room temperature for 48 hours prior to processing. They were dehydrated by immersion into 70%, 96% and 100% alcohol (twice for a period of one hour) and stored overnight in chloroform at 56 °C. The tissues were then placed into a mixture of chloroform and paraplast (1:1) for one hour at 56 °C, and then embedded in paraplast I and paraplast II (for one hour at the same temperature). A microtome was used to cut 6 μm-thick sections, which were then fixed onto slides with 2% APES (3-aminopropil-trietoxilene: Sigma, St. Louis, U.S.A.) in acetone. The sections were then cleaned of paraffin by immersion into xylol (2x10 min.), a succession of alcohol concentrations (5 minutes each in 100%, 96%, 80% and 70% concentration), and into distilled water (2 x 5 minutes). The next phase was staining with hemalaun-eosin and embedding into Canadian balsam. Thus prepared histological slides were studied using a light microscope.

Animals used in this study were maintained 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.

Statistical processing

The values obtained from studied indicators were processed using the general linear model procedure of the STATISTICA (data analysis software system), version 7.1. (StatSoft, Inc. (2005). Differences between the control and trial groups were statistically tested using repeated measurement model with Duncan's *post hoc* test.

RESULTS

Production indicators and clinical observations

At the end of the experiment body mass was similar in all groups, although lower body mass was determined in E2 group, but without statistical significance (Table 2). Weaned piglets from control groups (C1 and C2) did not show any signs of oestrogenism. Swelling of pudenda and red mammal complex were found in the group of piglets to which was administrated zearalenone (E2). Piglets from group E1 had lightly redened nipples.

Table 2 Body weight of weaned piglets fed on fodder mixture contaminated by zearalenone with addition of clinoptilolite Min-a-Zel Plus®

Groups	Body weight					
	Start			Final		
	N	$\bar{x}$	sd	N	$\bar{x}$	sd
C1	5	13.11	1.63	5	16.49	1.46
C2	5	13.68	1.55	5	16.93	1.76
E1	5	12.86	2.18	5	16.64	1.52
E2	5	12.71	2.05	5	15.70	2.60

C1 – control group, C2 – Min-a Zel Plus®, E1 – ZEN + Min-a-Zel Plus® , E2 – ZEN

Biochemical parameters

According to data presented in Table 3, glucose values in all groups during the observed period were within the parameters of physiological values (Kaneko, 1997). However, a significantly higher ($P<0.05$) value was established in the control group (C1) on the 8th day in relation to experimental E1 and E2 groups, and on the 14th day in relation to C2 and E2 groups. Comparing the values between groups it was found that on the 8th day piglets in the E1 group had lower levels of urea in relation to C1 group and higher levels on the 14th day in group C1 in relation to C2 group of piglets. The values of triglicerydes were significantly higher ($P<0.01$) in control group C1 in relation to all tested groups during both tested periods. The values of total proteins were significantly lower ($P<0.01$) in the control group C1 in relation to other tested groups. Differences between albumin concentrations were not significant, but the higher concentration was determined in C1 group. The level of iron in the serum was significantly lower ($P<0.01$) in the group of piglets given the feed mixture with added ZEN (E2) as early as day 8. This trend continued until the end of the experiment.

Peripheral blood count

Results of blood count monitored in the middle and at the end of the experiment show that there was no deviation from physiological values (Table 4). Nevertheless, on the 8th day the animals in control group C2 showed an increased level of leucocytes. The highest number of erytrocytes was in C1 group, while hemoglobin and hematocrit concentration were with no statistical significance between groups. MCV was also significantly higher ($P<0.01$) in the group of piglets which was given ZEN contaminated food (E2) in comparison with the group fed on feed containing Min-a-Zel Plus® and ZEN (E1) and control C1 group. Values of MCHC were significantly lower in C2, E1 and E2 group in comparison with C1 group. Number of trombocytes was significantly higher ($P<0.01$) in C1 group in comparison with all other groups during both periods of observation. Of the total number of leucocytes on the 8th day of the experiment the participation of segmented leukocytes was higher ($P<0.05$) in group E1 in relation to C1 group. Participation of lymphocytes and monocytes was higher in group E2, but no significant differences were established.

Table 3 Biochemical values in blood serum of weaned piglets on the 8th and the 14th day of trial fed on zearalenone contaminated feed in addition of clinoptilolite Min-a-Zel Plus®

Indicators	Days	Groups			
		C1[a] $\bar{x}$ ± SEM	C2[b] $\bar{x}$ ± SEM	E1[c] $\bar{x}$ ± SEM	E2[d] $\bar{x}$ ± SEM
Glucose, mmol.L^{-1}	8	5.33±0.37*E1,E2	4.30 ± 0.40	3.92 ± 0.45*C1	3.72 ± 0.40*C1
	14	5.05±0.37*C2,E2	3.58 ± 0.40*C1	4.17 ± 0.45	3.66 ± 0.40*C1
Urea, mmol.L^{-1}	8	4.53±0.40*E1	5.38 ± 0.43	6.17 ± 0.49*C1	5.22 ± 0.43
	14	5.20±0.40*C2	3.60 ± 0.43*C1	4.80 ± 0.56	4.35 ± 0.49
Creatinine, µmol.L^{-1}	8	62.33±7.77	69.80 ± 8.52	63.75 ± 9.52	70.40 ± 8.52
	14	61.16±7.77	45.80 ± 8.52	50.50 ± 9.52	62.50 ± 9.52
Bilirubin-total, µmol.L^{-1}	8	5.66±17.59	4.40 ± 19.27	4.75 ± 21.54	4.80 ± 19.27
	14	5.33±17.59	3.60 ± 19.27	3.75 ± 21.54	5.50 ± 21.54
Total protein, g.L^{-1}	8	48.25±2.52**C2,E1,E2	62.20 ± 2.76**C1	58.25 ± 3.09*E2, **C1	61.50 ± 2.76*E1,**C1
	14	48.51±2.52*C2,**E2	58.38 ± 2.76*C1	54.92 ± 3.09**E2	64.42 ± 2.76**C1,E1
Albumin, g^{-1}	8	34.15±2.77	28.84 ± 3.03	30.00 ± 3.98	27.80 ± 3.03
	14	33.45±2.77	27.88 ± 3.03	29.27 ± 3.98	29.86 ± 3.03
Cholesterol, mmol.L^{-1}	8	1.81±0.59	1.93 ± 0.65	1.53 ± 0.72	1.99 ± 0.65
	14	1.77±0.59	2.55 ± 0.65	2.71 ± 0.89	2.96 ± 0.65
Triglyceride, mmol. L^{-1}	8	1.18±0.07**C2,E1,E2	0.46 ± 0.08**C1	0.52 ± 0.09**C1	0.33 ± 0.08**C1
	14	0.95±0.07**C2,E1,E2	0.47 ± 0.10**C1	0.57 ± 0.10**C1	0.33 ± 0.09**C1
Fe, µmol L^{-1}	8	23.80±1.36**C2,E1,E2	12.08 ± 1.49**C1,E2	13.10 ± 1.66**C1,E2	4.80 ± 1.49**C1,C2,E1
	14	21.98±1.36**C2,E1,E2	8.33 ± 1.92**C1	7.66 ± 1.92**C1	4.67 ± 1.66**C1

C1 – control, C2 – Min-a Zel Plus®, E1 – ZEN + Min-a-Zel Plus®, E2 – ZEN ; * P<0.05; ** P<0.01
[a]N = 6, 6 for 8th and 14th day of the trial; [b]N = 5, 4 (3 for Urea, Triglyceride and Fe) for 8th and 14th day of the trial;
[c]N= 4, 4 (3 for Urea, Triglyceride and Fe) for 8th and 14th day of the trial; [d]N= 5, 4 for 8th and 14th day of the trial

Table 4 Haematological values of weaned piglets on the 8th and 14th day of trial fed on zearalenone contaminated feed in addition of clinoptilolite Min-a-Zel Plus® (n=5)

Indicators	Days	Groups			
		C1[a] $\bar{x} \pm$ SEM	C2[b] $\bar{x} \pm$ SEM	E1[c] $\bar{x} \pm$ SEM	E2[d] $\bar{x} \pm$ SEM
Leukocytes x10^9/l	8	17.63±2.77	24.36±3.03	22.02±3.39	23.78±3.03
	14	19.68±2.77	22.36±3.03	21.27±3.39	22.24±3.03
Erythrocytes x10^{12}/l	8	6.73±0.24	6.35±0.27	6.59±0.30	6.36±0.27
	14	6.35±0.24*E1	5.71±0.27	5.44±0.30*C1	5.73±0.27
Haemoglobin, g/L	8	116.30±7.54	115.20±8.26	110.25±9.24	112.80±8.26
	14	92.55±7.54	104.6 ±8.26	100.7 ±9.24	105.2 ±8.26
Haematocrit, L/L	8	0.36±0.01	0.42±0.02	0.41±0.02	0.42±0.02
	14	0.34±0.01	0.36±0.02	0.31±0.02	0.33±0.02
MCV, fl	8	54.05±1.16**C2,E1,E2	66.38±1.27**C1,E1	61.67±1.42**C1,C2,E2	65.68±1.27**C1,C2,E1
	14	54.10±1.16**C2,E2	63.80±1.27**C1,E1,E2	56.52±1.42**C2	58.80±1.27**C1,C2
MCH, pg	8	17.25±0.43	18.14±0.47	16.70±0.53	17.76±0.47
	14	17.16±0.43*E1	18.32±0.47	18.80±0.53*C1	18.46±0.47
MCHC, g/l	8	319.16±9.81*C2**E1,E2	273.60±10.75*C1	270.50±12.01*C1	270.20±10.75**C1
	14	317.66±9.81	287.00±10.75**E1	335.50±12.01**C2	315.80±10.75
Trombocytes x10^9/l	8	739.16±68.54**C2,E1,E2	330.80±75.08**C1	239.75±83.94**C1	299.40±75.08**C1
	14	759.16±68.54**C1,E1,E2	448.40±75.08**C1	446.25±83.94*C1	423.20±75.08**C1
Band Neutrophils,%	8	7.16±1.87	5.20±2.05	6.00±2.29	6.40±2.05
	14	11.50±1.87	5.80±2.05	5.00±2.29	6.20±2.05
Neutrophils, %	8	42.00±3.20*E1	50.80±3.50	55.00±3.91*C1	46.80±3.50
	14	49.50±3.20	49.00±3.50	47.25±3.91	49.20±3.50
Lymphocytes, %	8	45.00±3.09*E1	36.40±3.39	33.25±3.79*C1	40.00±3.39
	14	29.16±3.09*C2,E1,E2	40.20±3.39*C1	42.75±3.79*C1	40.80±3.39*C1
Monocytes, %	8	4.66±1.05	5.60±1.15	5.75±1.28	6.80±1.15
	14	8.50±1.05	5.00±1.15	5.00±1.28	5.80±1.15

C1 – control group, C2 – Min-a Zel Plus®, E1 – ZEN + Min-a-Zel Plus®, E2 – ZEN
[a]N = 6,6 for the 8th and 14th day of the trial; [b]N = 5,5 for the 8th and 14th day of the trial;
[c]N = 4,4 for the 8th and 14th day of the trial; [d]N = 5,5 for the 8th and 14th day of the trial

Table 5. Activity of enzymes in blood serum of weaned piglets on the 8th and 14th day of trial fed on zearalenone contaminated feed in addition of clinoptilolite Min-a-Zel Plus® (n=5)

Enzyme U·L^{-1}	Day	Groups			
		C1[a] $\bar{x} \pm$ SEM	C2[b] $\bar{x} \pm$ SEM	E1[c] $\bar{x} \pm$ SEM	E2[d] $\bar{x} \pm$ SEM
AST	8	24.66±5.99*^{E2}, **^{E1}	37.20±6.57**^{E1}	77.50±7.34**$^{C1, C2, E2}$	52.20±6.57*$^{E1, C1}$
	14	38.00±5.99**^{E2}	37.00±8.48**^{E2}	47.00±8.48**^{E2}	83.25±7.34**$^{C1, C2, E1}$
ALT	8	30.66±2.68	36.80±2.94	40.75±3.29	32.00±2.94
	14	33.83±2.68	36.75±3.29	30.33±3.79	36.75±3.29
ALP	8	182.33±16.86*^{E1}	153.20±18.47	109.75±20.65*^{C1}	134.80±18.47
	14	157.33±16.86*$^{C2, E2}$	78.50±20.65*^{C1}	103.33±23.84	71.75±20.65*^{C1}
GGT	8	26.66±5.05*^{E2}	45.20±5.53	39.75±6.18	48.60±5.53*^{C1}
	14	36.50±5.05	47.33±7.14	38.3 ±7.14	51.25±6.18
CHE	8	682.66±39.93	638.20±43.74	568.00±48.90	619.00±43.74
	14	706.33±39.93*$^{E1, E2}$	586.00±56.47	526.66±56.47*^{C1}	520.75±48.90*^{C1}
AMY	8	1640.33±199.09	1916.40±218.09	1392.75±243.83	1261.60±218.09
	14	1572.16±199.09	1484.50±243.83	1158.00±281.55	870.75±243.83
CK	8	175.83±293.83*^{E1}	233.60±321.88*^{E1}	1440.00±359.87*$^{C2, C1}$	615.20±321.88
	14	357.83±293.83**^{E2}	298.60±321.88**^{E2}	488.75±359.87**^{E2}	2162.40±321.88**$^{C2, C1, E1}$
LDH	8	378.00±194.64	785.00±212.22	752.25±238.39	823.80±213.22
	14	353.50±194.64*^{E2}	758.20±213.22	891.50±238.39*^{C1}	1078.20±213.22*$_{C1}$

AST – aspartate amino transferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase, GGT – gamaglutamyltransferase, CHE – cholinesterase, CK – creatine kinase, LDH – lactate dehydrogenase and AMY – amylase
C1 – control, C2 – Min-a Zel Plus®, E1 – ZEN + Min-a-Zel Plus®, E2 – ZEN
[a]N = 6, 6 for the 8th and 14th day of the trial; [b]N= 5, 4 (3 for AST; 5 for the CK) for the 8th and 14th day of the trial;
[c]N = 4, 3 (4 for CK and LDH) for the 8th and 14th day of the trial; [d]N = 5, 4 (5 for CK and LDH) for the 8th and 14th day of the trial

Enzyme activities

On day 8 AST activity was significantly higher ($P<0.01$) in group E1 in relation to both control groups (C1, C2), and on the 14th day the highest ($P<0.01$) activity was in E2 group. ALT activity was significantly higher ($P<0.05$) in the control group C1 in relation to E1 group on the 8th day, and on 14th day in relation to C2 and E2 group. A significantly higher ($P<0.05$) activity of GGT in the group of piglets fed with a higher ZEN content in relation to C1 group was determined. CK activity was significantly lower (on day 8 $P<0.05$ and on day 14 $P<0.01$) in both control groups, the highest activity was in E2 group on 14th day of experiment. LDH activity was significantly higher ($P<0.05$) in the group of piglets fed with the higher level of ZEN in comparison to control C1 group on day 14 (Table 5).

Histological findings

Table 6 shows that histological changes in group E2 affected sex organs (ovaries and uterus), lymphocyte depletion in lymph glands and interstitial inflammation of liver were established. In E1 group changes were confined to sex organs, while the control group manifested no pathological changes in the observed organs.

Table 6 Histological findings of weaned piglets fed on zearalenone contaminated feed in addition of clinoptilolite Min-a-Zel Plus® sacrificed on the 14th day

Histopatological findings	Groups			
	C1	C2	E1	E2
Hepatitis interstitialis	0/0	0/0	1/5	4/5
Depletio lymphocitaria lienis et lymphonodulli	0/0	0/0	2/5	5/5
Many secondary oocytes	0/0	0/0	0/0	5/5
Hyperplasia glandularis uteri	0/0	0/0	1/5	5/5
Primary follicles on ovaries	2/5	2/5	3/5	5/5

number of positive findings / total numbers of animals

DISCUSSION

The ability of zeolites to bind mycotoxins as well as heavy metals (Pond and Yen, 1983; Pond, 1985; Lindermann *et al*., 1993) was discovered and has been used for years. Other properties it possesses, i. e. not to bind amino acids, vitamins and minerals, as well as having no detrimental effect on the composition of the serum (Papaioannou *et al*., 2002), combined with a possible anti-carcinogenic effect (Martin-Kleiner *et al*., 2001, Pavelić *et al*., 2002) make it suitable for administration in the diet of domestic animals. A positive effect of the addition of organozeolite to the feed for lambs (Pond, 1985), broiler chickens (Dwyer *et al*., 1997) and better egg production in laying hens (Olver, 1997) has

been determined. Fokas *et al*. (2004) determined no effect of organozeolites on better nutrient utilization in growing pigs, but Papaiounnou *et al*. (2002) found it to be beneficial for sows, gilts and their litters' performance. Results about effects of clinoptilolite like a feed aditive, especially in feed contaminated with mycotoxins and heavy metals, applied in different experimental models, varied in daily gain and feed consumption. While Ward *et al.* (1991) and Pond *et al*. (1988) found possitive effects of clinoptilolite on growing pigs, Poulsen and Oksbjerg (1995) determined no differences on swine performance. In our experiment there are no significant differences in body weight between the groups. Group of piglets with increased levels of ZEN in food had lower body weights and their variability was higher. The negative effect of ZEN on the growth and feed conversion was established by Horugel and Vergara (2003), and Kalliamurthy *et al*. (1997). However, other authors regard it as a strong anabolic (Pfaffl *et al*., 2001). The observed changes on external sex organs and results of histological tests (Table 6) point to the already known agonistic oestrogenic effect of ZEN. The administration of clinoptiolite has not substantially reduced the ZEN effect but it was slightly weaker, which was manifest by the absence of secondary follicles on ovaries in group E1 animals and weaker simptoms of estrogenism in the same group. Research involving lambs showed that the compound clinoptilolite had a positive effect on the adsorption of mycotoxins, greater when the level of clinoptilolite was 0.5% than when it was 0.2% (Stojšić *et al*., 2004).

Literatures data point out that lower ZEN levels influence haematological parameters in animals. Gajecka et al. (2004) have established significant ZEN influence of haematological results with regard to the number of erythrocytes and leucocytes, haemoglobin concentration, values of haematocrit, values of MCH and MCHC and the share of segmented neutrophilic granulocytes and lymphocytes. Large quantities of absorbed ZEN, as recorded those in E2, reduced the MCHC values in relation to C1 group after 8 days of trial. Ivković *et al*. (2004) found that the natural zeolite clinoptilolite used as a dietary supplement did not relevantly affect blood count parameters, but Martin-Kleiner *et al*. (2001) determined a higher level of erythrocytes and leucocytes, especially lymphocytes and lower level of trombocytes in mice with when clinoptilolite was added. While Parentmassin and Parchment (1998) did not find ZEN to be haematotoxic, other authors confirmed that ZEN acts on the haematocrit, MCV, WBC and on the number of platelets (Maaroufi *et al*., 1996). The higher number of total leucocytes, higher lymphocytes and segmented neutrophyls level on the 14^{th} day of our study in the experimental groups in relation to control C1 group are in accordance with the investigation of Martin-Kleiner *et al*. (2001) on mice, Kececi *et al*. (1998) and Basmacioglu *et al*. (2005) on broiler chicken and Maaroufi *et al*. (1996) on rats. Values of MCV, MCH and MCHC, the changes of which were found in our investigation, are used for an early detection of the anaemia-causing process (Tyler i Cowell, 1996).

The effect of clinoptilolite and ZEN on biochemical parameters and enzyme activities in serum was also investegated. Hormones increase the influx of blood triacyglycerols into adipose tissue (Beitz, 2004). Oestrogens also increase synthesis of triacylglycerols and fat deposition (Goldfein and Monroe, 1997) and

lower the circulating levels of cholesterol and increase the level of very low-density lipoproteins, which results in an increase of the circulating levels of triacylglicerols (Ojeda, 2000). ZEN is a full agonist for estrogen receptors (ERα) and a mixed agonist-antagonist for ERβ and only a very small difference in the binding affinity of zearalenone to ERα and ERβ is detectable (Kuiper *et al*., 1998). In E2 group antioestrogenic metabolic effect of ZEN on the metabolism of triacylglicerols and cholesterol was determined, which showed significantly ($P<0.01$) lower triacylglicerols level and higher cholesterol level on day 14. In this group a characteristic response of the liver to the effect of oestrogen activity was determined (model such as raloxifene, a selective estrogen receptor modulator, Heringa, 2003). Having in mind that piglets from group E1 had the highest urea level and a lower level of total proteins in relation to E2, it can be assumed that increased triacylglycerol levels in group E1 were accompanied by intensified lipogenesis from carbohydrates. On day 8 group E1 had the highest urea level, with a concurrent increased activity of AST and ALT which, in combination with the highest glucose value on day 14, would indicate increased glucogenesis and increased synthesis of fats in the liver. This kind of effect would be similar to the effect of the coumestrol phytoestrogen (Nogowski, 1999; Nogowski *et al*., 2002) and could not be attributed to the oestrogen activity pattern of zearalenone. It was found that zearalenone was a potent estrogen and activated the preferentially oestrogen receptor alpha and was antagonistic on both oestrogen receptors alpha and beta at high doses (Mueller *et al*., 2004). The opposite effect of zearalenone is also visible in the increase of total proteins in E2, caused by a direct increase in the globulin fraction on day 14 of the experiment. The powerful influence that ZEN has on the lowering of serum iron, which was found to be significantly lower ($P<0.01$) in group E2 as early as day 8, can be explained through the inhibition of transferrin synthesis, the major Fe-transport protein in the plasma that is synthesized by the hepatocytes. Phytoestrogens and mycoestrogens act as weak mitogens for breast tumor cells *in vitro*, compete with 17β-estradiol for binding to ERα protein and induce activity of estrogen-responsive reporter gene in the presence of ERα protein (Mäkelä *et al*., 1994).

ZEN has affected CK activity, causing its increase. Its significant increase was determined in E1 group in relation to both groups C1 and C2 on day 8 and on day 14 the same occurred in group E2. Based on the distribution of oestrogen receptors a high ERα expression could be observed in the uterus, udder and liver, but also in muscles (Pfaffl *et al*., 2001). Analyses of muscle tissue revealed relatively high amounts of nonglucoronidated zeranol and α-zearalenol, together with traces of taleranol and zearalenone, indicating that the metabolism of zearalenone and its metabolites is not restricted to hepatic and gastrointestinal metabolic pathways (Zöllner *et al*., 2002). Early uterotrophic uterine responses also include the oestrogen-induced expression of creatine kinase (Pentecost *et al.*, 1990). Brain isoenzymes of CK represented 73% of total activity of skinned guinea-pig uterus (Clark *et al*., 1993). The rapid stimulation of the specific activity of the brain-type isoenzyme of creatine kinase is an almost universal marker of cell stimulation. The increase in its activity is closely correlated with the biochemical and morphological parameters, and the increase in CK activity has been used to

demonstrate specific stimulation by oestrogens in the skeletal-derived cells (Kaye *et al*., 1997), uterus adipose tissue (Somjen *et al*., 1996), vascular smooth muscle cells (Somjen *et al*., 2002; Somjen *et al*., 2004). Phytoestrogens have oestrogen-mimetic effects on cell growth and CK in the cultured human vascular cells and on the CK in rat vascular tissues *in vivo*, and the effects on replication are highly dependent on CK concentration (Somjen *et al*., 2001). Cholinesterase activity was significantly lower ($P<0.05$) in group E1 and E2 in relation to C1 as a consequence of cholinesterase inhibiting caracteristic for oestrogen activity (Bullock *et al*., 2002). Increased LDH activity is a predictive factor for central nervous system metastasis in patients with metastatic breast cancer (Ryberg *et al*., 2005) which is in relation to the oestrogen effect of ZEN.

Conclusions: It is determined that ZEN and clinoptilolite influence haematological parameters and serum chemistry. ZEN acts as a full agonist for ERα and mixed agonist-antagonist for ERβ receptors. Oestrogenic effect of ZEN was evident in its sinergistic action with insulin, which was manifested in an increase in total protein level, lower level of glucose, triacylglycerols, serum iron, a higher level of cholesterol and higher aminotransaminase activity. The metabolic antioestrogen effect of ZEN was established in group E2, whereas group E1 manifested the agonistic effect due to the oestrogen reduction by clinoptilolite.

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HEMATOLOŠKI I BIOHEMIJSKI PARAMETRI KOD ZALUČENE PRASADI HRANJENE KONCENTRATOM KONTAMINIRANIM ZEARALENONOM UZ DODATAK KLINOPTILOLITA

ŠPERANDA MARCELA, LIKER B, ŠPERANDA T, ŠERIĆ V, ANTUNOVIĆ Z, GRABAREVIĆ Ž, SENČIĆ Đ, GRGURIĆ D i STEINER Z

SADRŽAJ

U radu je istraživan utjecaj zeolita klinoptilolita (CLIN) na hematološke i neke metaboličke pokazatelje u serumu odbite prasadi hranjenje hranom s povišenom razinom zearalenona (ZEN) tijekom 14 dana. Istraživanje je provedeno na četiri skupine odbite prasadi u dobi od 40. do 54. dana. Sve su skupine hranjene krmnom smjesom za prasad u porastu, koja je sadržavala 20% sirovih proteina i 12.57 MJ ME/kg. Prva kontrolna skupina (C1) životinja hranjena je smjesom bez dodatka adsorbenta mikotoksina Min-a-Zel Plus® i bez dodatka zearalenona. Druga kontrolna skupina (C2) hranjena je hranom u koju je umješano 0.2% modificiranog klinoptilolita (Min-a-Zel Plus®; razina ZEN < 5.1 ng/g). U hranu za prasad pokusne skupine (E1) dodano je 3 mg/kg zearalenona (Sigma-Aldrich Co) i 0.2% pripravka Min-a-Zel Plus®. Drugoj pokusnoj skupini (E2) u hranu je umiješano 3 mg/kg zearalenona. Estrogeni efekt zearalenona očitovao se u sinergističnom djelovanju s inzulinom, što se očitovalo povišenom razinom ukupnih proteina u serumu, nižom razinom glukoze, triglicerida, serumskog željeza, povišenom razinom kolesterola i višom aktivnosti transaminaza. Metabolički antiestrogeni učinak ZEN utvrđen je u E2 skupini, dok je u E1 skupini utvrđen agonistički efekt ZEN zbog smanjene resorpcije zahvaljujući djelovanju klinoptilolita.