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EFFECT OF COCCIDIOSIS ON THE ENZYMATIC AND NON-ENZYMATIC ANTIOXIDATIVE SYSTEMS IN BROILERS

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This report describes an investigation of the effects of coccidial infection on enzymatic and non-enzymatic antioxidative systems in hemolyzed blood and liver homogenate from broilers (glutathione - GSH, glutathione reductase - GSHR, glutathione peroxidase - GSH Px, peroxidase - Px, superoxide dismutase - SOD, xanthine oxidase - XOD and lipid peroxidation - LPx).

The in vivo investigations were carried out on heavy-line broilers (Arbor acres) of both sexes. One day old broilers were randomly distributed into two groups each amounting to 60 chickens: I - control group; II - broilers inoculated with laboratory - derived coccidia species.

Inoculation of 22-day old broilers of the experimental group II was performed by p. o. administration of a coccidial suspension containing 2.000 oocysts of Eimeria (E. tenella, E. mitis, and E. necatrix). Decapitation and blood sampling was performed when the control chickens were 32 days old and in the experimental group when symptoms of coccidiosis appeared.

It was concluded that infection of the broilers with coccidia intensifies free radical processing in hemolyzed blood and liver homogenate. This was evident from the increased levels of GSH and LPx catalytic activity of almost all tested enzymes. Changes in the level of enzymamtic activity were more pronounced in the blood than in the liver of the infected birds.

Key words: broilers, antioxidative system, Eimeria spp, heolyzed blood, liver homogenate.

INTRODUCTION

The genus Eimeria comprises a population of obligate intracellular protozoan species with a complex life-cicle including both asexual and sexual stages of development. Coccidiosis is the consequence of Eimeria infection of the intestine of the permissive host. This condition markedly reduces growth and feed utilization in poultry and livestock. Due to intensive breeding of poultry, coccidiosis is a lingering economical problem for poultry industries worldwide. Coccidiosis is traditionally controlled by chemotherapy, but the persistent appearance of drug-resistant strains of coccidia calls for the development of alternative strategies.

The basic assumption for monitoring biochemical parameters in the group of experimentally infected broilers was that the pathological condition, caused by coccidiosis, will most likely induce changes in enzymatic and non-enzymatic antioxidative systems of the affected animals, and therefore data about the infection could be obtained prior to the appearance of clinical symptoms. If the assumption proves to be true, a possibility arises for timely diagnosis and adequate therapy. This could help intensive poultry farming by reducing economic loss due to poor food conversion and the extermination of entire flocks.

In pathobiochemistry, free radicals that appear during oxidative stress (e. g. illness, environmental pollution) may cause diverse dellular damage. Free radicals are defined as atoms or molecules bearing one or more unpaired electrons that make them highly reactive (Gruber, 1994). Molecular species of biological importance are the following: superoxide radicals, hydroxyl radicals and derived molecules such as hydrogen peroxide, lipid peroxide and highly reactive singlet oxygen (Brawn and Fridovich, 1982).

Since the very existence of free radicals is an indispensable constituent of all aerobic cells, in the course of evolution many different mechanisms have emerged that eliminate these molecular species or reduce their harmful effects. The studyof these processes revealed great complexity and interplay, so all physiological defense mechanisms are classified as the system of antioxidative protection. These protective mechanismms include enzymatic, non-enzymatic and secondary antioxidative protection.

The enzymes involved are:

- Superoxide dismutase (SOD) EC 1. 15. 1. 1.
- Glutathione peroxidase (GSH Px) EC 1. 11. 1.9.
- Glutathione S-transferase (GST) EC 2, 5. 1. 18.
- Glutathione reductase (GSHR) EC 1.6.4.2.
- Catalase (CAT) EC 1. 11. 1. 6.
- Peroxidase (Px) EC 1. 11. 1. 7.
- Xanthine oxidase (XOD) EC 1. 2. 3. 2.

Determing the catalytic activity of an enzyme for diagnostic purpose in blood diseases and different tissue disorders is particularly important for hereditary and acquired defects involving the enzymes of erythrocytes. Erythrocytes are prominent among cells in terms of the complexity of the

response to oxidative stress. They are directly exposed to molecular oxygen, their plasma membrane has high levels of polyunsaturated fatty acids and an anionic channel specific for singlet oxygen. Erythrocytes contain a high concentration of hemoglobin that isprone to auto-oxidation (Jovanović, 1993). They have a very efficient protective system that maintains them in circulation and enables them to perform normal functions. This system implies a high level of anti-oxidant protection and GSH (Prins and Loss, 1969).

The erythrocyte defense from reactive oxygen radicals involves four enzymes. The first two, acting synergistically are CAT and GSH Px. They are coupled in the removal of hydrogen peroxide. Normally, they compete for the most prominent oxidative agent in the maintenance of erythrocyte function. Normal erythrocytes contain two hemoglobin reductases continuously regenerating Hb, providing specific coenzymes: $NADH_2$ from glycolysis and $NADPH_2$ from the hexosemonophosphate pathway (Mimić-Oka, 1994).

The liver is an organ with a central metabolic role in the organism, often referred to as "the main laboratory" since it performs the major detoxification tasks. Diverse mechanisms are involved. For this reason the liver is the prime target for study of the metabolism of xenobiotics. Thus, liver homogenates, thin sections of the tissue, perfused liver, suspensions and cultures of hepatocytes and microsomal preparations are usually utilized as model system. (Popović, 1988.). It has been demonstrated that lipid peroxides, accumulated in other tissues, are transported to the liver for further processing. For this reason, enzymes for anti-oxidative protection, GSH Px and GST in particular, are essential for normal liver physiology (Little, and O'Brien, 1968).

Among all the organs, the liver contains the highest lever of GSH (5-10 mmol/g) which is presumed to be the major defense mechanism against xenobiotics, normally excreted as conjugates with GSH. The second important role of GSH is protection against organic peroxides via GSH Px. Reduced levels of GSH in isolated hepatocytes result in the accumulation of H_2O_2 (Kaplowitz et al., 1985). Elevated peroxidation of liver lipids was demonstrated as the consequence of reduced levels of GSH, both *in vivo* and in isolated hepatocytes. This is additional support for the protective role of GSH Px against endogenous peroxides (Popović, 1988).

MATERIALS AND METHODS

Experiments under *in vivo* conditions were performed on broilers of the heavy line, Arbor acres, of both sexes. One-day-old broilers, randomly selected, were divided into two groups, each numbering 60 individuals: I - control group; II - experimental group inoculated with laboratory strains of coccidia.

Inoculation of 22 day old broilers was performed by p. o. application of 1 cm^3 of coccidial suspension containing 2000 oocysts of *Eimeria (E. tenella, E. mitis, and E. necatrix)*. Blood sampling and sacrifice in the control group was at 32 days of age, while in the experimental group, these procedures were performed when symptoms of coccidiosis appeared.

Levels of hemoglobin, necessary for the expression of the enzymatic activities in hemolyzed blood, were determined using commercial test ("Dialab", Vienna, Austria) on a spectrophotometer ("Multiscan MCC340, Finland. Protein content was determined by the modified method of Gornall Bardwall, (1949).

In hemolyzed blood and homogenised liver, glutathione content, products of lipid peroxidation and the activities of anti-oxidant enzymes (SOD, GSHR, GSHPx, Px, and XOD), were determined.

Preparation of blood hemolysate. Blood was drawn from the hearts of broilers into heparinized test tubes. After centrifugation (10 min at 3500 rpm and 4^0) and plasma removal, the pellet was rinsed 3 times in saline. The resulting erythrocyte pellet was suspended in an equal volume of double distilled of water and vortexed. After incubation for 1 hour at room temperature, the hemolysate was centrifuged for 15 min at 3500 rpm and the supernatant aliquoted for further analysis.

Preparation of liver homogenate. The excised liver was perfused to eliminate blood and the total mass determined. One gram of the tissue was minced with scissors and homogenized in an ultratorax in 3 volumes of isotonic buffer (0.05M Tris-HCl, 0.25M sucrose, pH=7.5). The homogenate was filtered through gauze into ice-cold tubes and aliquoted for further analysis.

Sample preparation for GSH determination. In freshly prepared hemolysate, proteins were separated by adding half the volume of 10% sulfosalicylic acid and centrifugation at 5 000 rpm, for 5 min, at 4^0C. The supernatant was stored at 4^0C, without freezing, and GSH determined within 24 hours. The same procedure was applied for liver homogenate.

Determination of enzymatic activity. The SOD activity was determined by the spectrophotometric method based on the inhibition of adrenaline reduction to adrenochrome at pH = 10.2 (Jovanović, 1993).

The GSH Px activity was determined by spectrophotometric measurement of absorbance at 412 nm with cumenhydroperoxide as the substrate (Chin et al, 1976).

According to the method of Glatzle and coworkers (1974) the GSHR activity was determined from the rate of NADPH oxidation, monitored by the absorbance at 340 nm.

Lipid peroxidation was determined with thiobarbituric acid. The oxidation of cellular membrane lipids was measured via reaction of lipidperoxides with thiobarbituric acid (Buege and Aust, 1978).

The determination of Px activity was based on the catalytic oxidation of guayacole by hydrogen peroxide as an electron acceptor (Simmon et al., 1974).

The reaction of xanthine oxidation touric acid was used for determination of XOD activity (Bergmayer, 1970). Spectrophotometric measurement was performed in 0.1 mM phosphate buffer, pH=7.5, at 295 nm.

The GSH content in the blood hemolysate and the liver homogenate was determined from the amount of sulfhydryl residues by means of Ellmann's reagent, (Kapetanović, and Mieyal, 1979). The erythrocyte content of GSH was determined by the modified method of Beutler et al.(1963) and in the liver homogenates by the modified of Ellmann (Kapetanović,and Mieyal, 1979).

Statistical processing of the results was performed by analysis of variance.

RESULTS AND DISCUSSION

The GSH and LPx levels and enzymatic activity found in blood hemolysate from the control group (32 days of age) and the experimental group are shown in Table I. These parameters of antioxidative protection in hemolysates of blood and homogenates of liver of broilers in the control group confirmed the findings of others (Salyi et al., 1990, 1992).

Table 1. GSH and LPx content and the activity of GSHPx,Px, SOD, GSHR and XOD in blood hemolysate

Parameter	I	II
GSH (μmol/g Hb)	5.30 $\pm$ 1.21	6.72 $\pm$ 2.11*
LPx (μmol/g Hb)	0.41 $\pm$ 0.09	5.34 $\pm$ 0.19**
GSHPx (μmol/g Hb min)	8.18 $\pm$ 2.39	12.92 $\pm$ 6.75*
Px (μmol/g Hb min)	64.78$\pm$ 3.87	95.17 $\pm$ 5.78*
SOD (μmol/g Hb min)	55.39 $\pm$ 7.00	61.35 $\pm$ 2.27*
GSHR (μmol/g Hb min)	19.38 $\pm$ 3.93	15.92 $\pm$ 6.08
XOD (μmol/g Hb min)	27.06 $\pm$ 1.88	14.96 $\pm$ 6.58*

I - the control group after 32 days of fattening
II - the group of artificially infected broilers
*- $LSD_{0.05}$, statistically significant
** - $LSD_{0.01}$, statistically very significant

The results presented in Table 1. indicate a statistically significant increase in the content of GSH and higher catalytic activity of almost all examined enzymes (GSHPx, Px and SOD) in blood hemolysates of infected broilers, while the increase in the content of LPx was very significant. The only exception was the catalytic activity of XOD which showed a statistically significant reduction compared with the control.

The most likely explanation for the observed phenomena is that the pathological alterations intensify free radical processes by stimulating catalytic activities of enzymes involved in the anti-oxidative protection, Px and SOD. The increased liposys in fatty deposits, in the course of the disease, when food intake is reduced and the organism exhausted by frequent hemorrhaging diarrhea, presents a protential danger for the increase of lipid peroxides in blood. For this reason the increase in LPx is not entirely unexpected. Concomitantly with the increased risk of peroxidation of lipids in blood, there is an increase in the enzymatic activity of GSHPx.

The content of GSH and LPx and the catalytic activity of selected enzymes of the anti-oxidative defense system found in the liver homogenates of the control and the experimental group are shown in Table 2.

Table2. Content of GSH and LPx and activity of GSH Px, SOD, GSHR and XOD in liver homogenates

Parameter	I	II
GSH (μ mol/mg of protein)	0.57 ± 0.12	1.28 ± 0.21**
LPx (μ mol/mg of protein)	0.40 ± 0.14	0.35 ± 0.19
GSHPx (μ mol/mg of protein per min)	4.80 ± 2.62	7.58 ± 2.58*
Px (μ mol/mg of protein per min)	5.67 ± 1.49	2.79 ± 0.89**
SOD (μ mol/mg of protein per min)	10.77 ± 4.83	9.78 ± 3.98
GSHR (μ mol/mg of protein per min)	31.67 ± 12.39	21.50 ± 5.19*
XOD (μ mol/mg of protein per min)	20.36 ± 8.77	18.63 ± 2.99

I the control group after 32 days of fattening
II - the group of artificially infected broilers
* - $LSD_{0.05}$, statistically significant
** - $LSD_{0.001}$, statistically very significant

In the liver homogenates of infected broilers (Table 2) the content of GSH and catalytic activity of GSHPx were significantly higher than the control values, while the activities of Px and GSHR were reduced.

Comparison of the results from Tables 1 and 2, showing the anti-oxidative protection in hemolyzed blood and liver homogenates of infected broilers, indicates good correlation in terms of the content of GSH and the catalytic activities of GSHR and XOD. The reduction of the catalytic activity of GSHR in the liver homogenates of infected chickens was statistically significant, while in the blood hemolysates, the slight reduction of this activity was without statistical significance. Moreover, the reduction of the activity of XOD in blood hemolysates and liver homogenates of infected chickens was statistically significant only in the blood hemolysates.

The results in Tables 1 and 2 also indicate more pronounced changes in the content and the activity of the non-enzymatic and enzymatic anti-oxidative protective system in blood hemolysates compared to those in the liver homogenates of infected chickens.

The finding of more pronounced alterations of the enzymatic activity in blood hemolysates than in liver homogenates of infected animals is in accordance with published data (Delić, 1962). When *Eimeria* enter the digestive system, different developmental stages secrete specific metabolities that may be absorbed and induce changes in the enzymatic activity of the anti-oxidative protective system in a variety of tissues including the liver and hematopoietic tissue.

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UTICAJ KOKCIDIOZE NA NEENZIMSKI I ENZIMSKI ANTIOKSIDATIVNI SISTEM BROJLERA

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SADRŽAJ

U radu je ispitan uticaj infekcije kokcidijama na neenzimski i enzimski antioksidativni sistem hemolizata krvi i homogenata jetre brojler pilića (glutation-GSH, glutation reduktaza-GSGR, glutation peroksidaza-GSH Px, peoksidaza-Px, superoksid dismutaza- SOD, ksantin oksidaza-XOD i peroksidacija lipida- LPx).

Ispitivanja u in vivo uslovima izvedena su na brojlerima teške linije. Arbor acres, oba pola. Jednodnevni pilići, metodom slučajnog izbora, podeljeni su u dve grupe, svaka po 60jedinki: I - brojleri kontrolne grupe: II - brojleri inficirani laboratorijski održavanim vrstama kokcidija.

Inficiranje 22. dnevnih pilića u eksperimentalnoj grupi II, izvršeno je peroralnim davanjem 1cm^3 suspenzije kokcidija, koja je sadržavala 2000 oocisti Eimeria (E. tenella, E. mitis i E. necatrix). Uzimanje uzoraka krvi i žrtvovanje u kontrolnoj grupi - I, izvršeno je 32 dana starosti, a u grupi veštački inficiranih brojlera - II, pri pojavi prvih kliničkih znakova kokcidioze.

Utvrđeno je da infekcija brojlera kokcidijama intenzivira slobodnoradikalske procese u hemolizatima krvi i u homogenatima jetre, što se zapaža iz povećanja sadržaja GSH i LPx i katalitičke aktivnosti skoro svih ispitivanih enzima u odnosu na kontrolnu grupu. Promene katalitičke aktivnosti enzima su izraženije u hemolizatorima krvi u odnosu na homogenate jetre veštački inficiranih brojlera.