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BLOOD SERUM PROTEIN STATUS IN BROILERS FED WITH INCREASING CONCENTRATIONS OF OCHRATOXIN A

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The 42-day long study was performed on a total number of 48 Hybro broilers divided into four groups. After the pre-experimental period of 14 days, 3 experimental groups of broilers (n=12) were formed and fed diets that contained 0.5, 1.0 and 1.5 mg/kg ochratoxin A (OTA) during the next seven consecutive days. In the same period, the control group of broilers was fed a diet with no toxin added. After the period of toxin addition, blood samples were taken from 6 animals in each group. The remaining animals (n=6) from the control and experimental groups were fed diets without OTA until the 42nd day of the study, when the blood samples were taken again. The total level of blood serum proteins was affected by treatment with different doses of OTA, but a significant and dose dependent increase of albumins together with a decrease of γ-globulin fraction was established. A/G ratio (Albumine/Globuline) suggested that the globulins were the dominant protein fraction in the blood serum samples obtained from all the broilers included in this study. The concentrations of α- and β-globulin in the serum were within physiological limits, but the concentration of γ-globulins significantly decreased. It can be concluded that the increasing dietary OTA levels (0.5, 1.0 and 1.5 mg/kg) had dose-dependent cumulative effect on blood serum proteins status in broilers, and the effect lasts even after the withdrawal of OTA from the feed.

Key words: A/G ratio, broiler, ochratoxin A, poultry, proteins

INTRODUCTION

Ochratoxins are highly toxic compounds commonly produced as secondary metabolites by two species of fungi: *Penicillin verrucosum Dierckx* and *Aspergillus alutaceus* (Harris and Mantle, 2001; Reverberi *et al*., 2010). The toxin is represented by three similar compounds, of which ochratoxin A ($C_{20}H_{18}ClNO_6$) is the most toxic and abundant. In recent years ochratoxin A (OTA) has drawn a considerable attention because it can not only seriously affect animal performance and health, but it may also have deleterious effects on humans. Of

greatest concern for the human population is its implied role in an irreversible and fatal kidney disease (Balkan endemic nephropathy) (Castegnaro *et al.*, 2006).

Ochratoxin A is a natural contaminant of poultry and livestock feedstuffs. Field out-breaks of ochratoxicosis have been documented (Danike, 2002; Hamilton *et al*., 1982) and experimental feeding of OTA was shown (Kubena *et al.*, 1985a; Devegowda and Aravind, 2002; Binder *et al*., 2007) to have detrimental effects on growing chicks, indicating that OTA is a potential hazard to poultry producers. The diagnosis of ochratoxicosis may be difficult. In the absence of specific, well-defined clinical signs (Humphreys, 1985) the most common effects were poor condition, lower performance or decreased production. Ochratoxin A exposure in poultry can cause reduced body weight gain (Kubena *et al.,* 1984), decreased feed consumption and diminished egg production (Prior and Sisodia, 1978), listlessness, huddling, occasional diarrhea, ataxia, prostration and finally death (Prior *et al.*, 1976) together with atrophy and degeneration of the tubules and interstitial fibrosis of the kidneys (Nedeljković Trailović *et al*., 2001). The biochemical effects of ochratoxin in poultry include the decrease in serum total protein concentration, as a major clinical observation. Many authors (Bailey *et al*., 1990; Kubena *et al*., 1981; Kubena *et al.,* 1985a) have described the negative effect of higher dietary OTA level (2.5-4.0 mg/kg) on broiler protein status, protein fraction and A/G ratio in the blood serum of broilers. On the other hand, there is limited data about the effect of dietary OTA levels below 2 mg/kg or less that are most frequently detected in feedstuffs (Nedeljković Trailović *et al.,* 2000).

The effects of 2 mg/kg dietary OTA on blood serum protein concentration were described by Kubena *et al.* (1984). The authors state that compared to controls, total serum protein and albumin concentrations, as well as A/G ratio was significantly reduced. These data are consistent with several earlier reports. Kubena *et al.* (1994) described a significant decrease of blood serum total protein and albumin, as well as A/G ratio in broilers treated with 2 mg/kg OTA during three weeks. Singh *et al.* (1990) fed broilers with 0.5 or 2.0 mg/kg dietary OTA during three weeks, but significantly decreased blood serum levels of total protein, albumin and globulin were confirmed only after treatment with higher dosage of OTA. Similarly, Huff and Ruff (1981) reported a significant decrease of blood plasma total proteins only after treatment with OTA levels above 2.0 mg/kg, while lower levels (0.5 or 1.0 mg/kg) had no significant effect on blood protein concentration.

Ayed *et al.* (1991) examined the influence of low dietary OTA level (0.5 mg/kg) on blood protein status. Broilers were fed the experimental diets for 4 weeks after which the rations were withdrawn and replaced by control finisher diets for 3 week recovery period. The concentration of total serum proteins was significantly decreased in the treated group, but after the recovery period, there were no significant differences in total serum proteins. This finding is not in agreement with the results obtained by Chang *et al.* (1981) who examined the effects of graded levels of dietary OTA up to 8 mg/kg and claimed that total plasma proteins were not altered by any of the treatments.

Although very extensive literature was published on the changes of blood serum proteins caused by OTA, the effects were still poorly understood and not

fully explained. The present study was designed to assess the effect of short-term treatment with increasing levels of dietary OTA on blood serum protein status of broilers, and to provide data on whether the potential effects of ochratoxin is dose dependent.

MATERIAL AND METHODS

Animals. Hybro broilers (n=48) were obtained from a commercial hatchery. The study was performed on one day-old male chickens, which were housed in wire floor battery brooders. Light cycle, temperature and moisture were maintained throughout the study according to the procedure described by Allameh *et al.* (2005).

Experimental design. After 14 days of pre-experimental period during which the animals were fed the usual diet without toxin, 48 broilers were allocated for the study. Animals were divided randomly into three experimental groups and one control group. Experimental groups were fed diets that contained increasing concentrations of OTA 0.5, 1.0 or 1.5 mg/kg, during seven consecutive days. After this period, broilers were fed a meal without addition of OTA until the end of the study (42nd day).

Diet. All broilers were fed the commercial concentrate mixtures that consisted of standard feedstuffs and contained enough nutrients to provide the requirements for this category. The composition of the diet was adjusted according to the National Research Council Recommendation (NRC, 1994). In the diet, the 99% pure ochratoxin A (Sigma-Aldrich, Inc., St. LOUIS, MO, USA), obtained from *Aspergillus ochraceus* culture (CAS 303-47-9) was added in the amount sufficient to provide 0.5. 1.0 and 1.5 mg of toxin per one kg of feed, respectively.

Data and sample collection. Blood collection was carried out at two time intervals. One set of broilers (n=24) was sacrificed at the age of 21 days and the other set was sacrificed at the age of 42 days (n=20). Four chickens have died between the 21st and 42nd day of the trial. Blood in the amount of 10 mL was taken by cardiac puncture with a sterile needle and on a previously disinfected injection site, immediately after the end of toxin administration period (21st day). Blood samples were taken from six broilers from each group and the remaining animals (n=6) from the control and experimental groups were normally fed with commercial mixture without added OTA until the 42nd day of the study, when the blood samples were taken again. After coagulation of blood and separation of blood serum, total protein concentration was determined by biuret colorimetric method. The relative amount of blood serum protein fractions was obtained after their separation by paper electrophoresis (Majkić-Singh and Spasić, 1990; Singh *et al*. 1990).

Statistical analysis. All data were statistically processed using Graf Prism Pad 4.0. Values are expressed as means and standard deviation of the mean (mean ± SD). All results were statistically tested by one-way analysis of variance (ANOVA) test, Student-t test and linear regression [(Effect) = a (y-intercept ±

S.E.M.) + b (slope ± S.E.M) · X (dose)]. While, differences were considered significant at $p<0.05$.

RESULTS

Wide physiological limits of blood serum protein concentrations followed by variations of their fractions were confirmed in the current study. Increasing concentrations of OTA in broilers feed had no effect on total protein concentrations in the blood serum (Table 1), but there was a change in the relation between concentrations of albumins and globulins.

Table 1. The influence of increasing feed concentrations of ochratoxin A on broilers blood serum concentration of total proteins, albumins, globulins [g/L] and A/G ratio

Group	n	Concentrations, g/L (X ± SD)			A/G Ratio
		Total proteins	Albumins	Globulins	
		21st day			
Control	6	33.79 ± 1.54	13.19 ± 0.85	20.60 ±1.79	0.65 ± 0.09
0.5 mg/kg	6	32.96 ± 2.32	12.19 ± 1.78	20.77 ±1.54	0.59 ± 0.10
1.0 mg/kg	6	33.31 ± 3.63	14.21 ± 1.21 o (p=0.0443)	19.11 ±2.65	0.76 ± 0.15 o (p=0.0435)
1.5 mg/kg	6	32.46 ± 2.25	14.30 ± 1.11 o (p=0.0335)	18.16 ±1.58 * (p=0.0395); o (p=0.0159)	0.79 ± 0.08 * (p=0.0173); oo (p=0.0033)
		42nd day			
Control	6	35.12 ± 2.40	11.92 ± 3.47	23.21 ± 2.21	0.53 ± 0.21
0.5 mg/kg	4	36.85 ± 1.12	17.71 ± 0.83 * (p=0.0173)	19.14 ± 1.66 * (p=0.0142)	0.93 ± 0.12 ** (p=0.0092)
1.0 mg/kg	4	34.02 ± 1.79	14.96 ± 0.62 oo (p=0.0018)	19.06 ± 1.38 * (p=0.0107)	0.78 ± 0.04 * (p=0.0499)
1.5 mg/kg	6	36.08 ± 2.89	17.67 ± 1.41 ** (p=0.0055); oo (p=0.0073)	18.08 ± 1.96 * (p=0.017)	0.97 ± 0.16 ** (p=0.0022)

* – comparison with control group; o – comparison with 0.5 mg/kg group

This finding directly confirms changes in A/G relationship where a significant increase compared to the control value in the period of treatment with OTA was observed, as well as the recovery period without the toxin (Table 1). The concentration of albumins in the serum of treated broilers has increased during the seven-day treatment, while OTA showed a dose-dependent effect (Figure 1). The most notable significant increase in the concentration of albumins was caused by OTA concentration of 1.5 mg/kg of feed (Table 1, Figure 1).

After a period of recovery without adding OTA to the feed (42nd day), the concentration of albumins was still elevated compared to the control findings, but without a clear dose-dependence (Table 1, Figure 1).

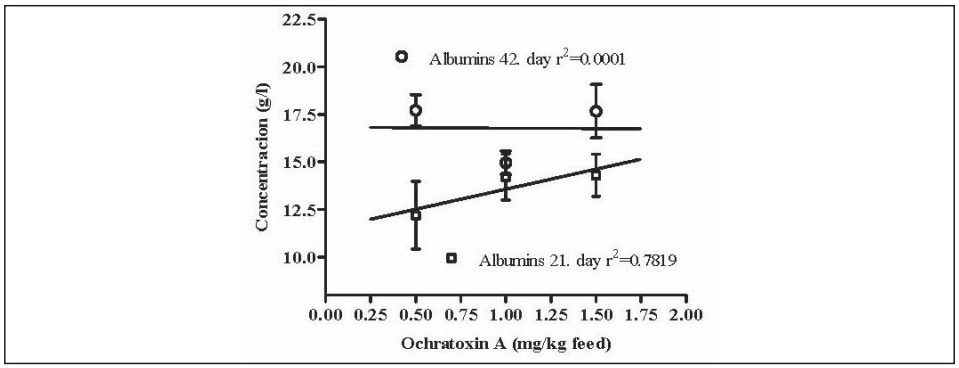

Figure 1. Effect of increasing concentrations of OTA (0.5, 1 and 1.5 mg/kg of feed) on the concentration of albumins in the blood serum of treated broilers

In contrast to the increasing concentration of albumins, the concentration of total globulins dose-dependently declined in broilers treated with OTA (Table 1, Figure 2).

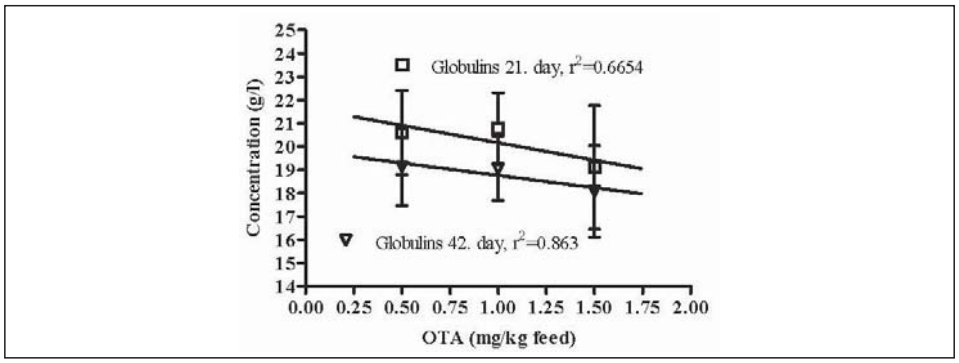

Figure 2. Effect of increasing concentrations of OTA (0.5, 1 and 1.5 mg/kg of feed) on the concentration of globulins in the blood serum of treated broilers

The decline of the concentration of globulins in relation to the control is continued in the recovery period (21st to 42nd day without toxin), therefore the dose-dependent effect of the toxin can be seen after 42 days of observation (Table 1, Figure 2). On the other hand, OTA in feed has led to specific changes in the relations between concentrations of serum globulin fractions of treated broilers. The changes are primarily related to the dose-dependent decrease in the concentrations of gamma-globulins, while the concentration of alpha and beta-

globulins are more or less unchanged, or changes are not reached the level of statistical significance (except for β-globulins which are significantly elevated in broilers treated with 1.5 mg/kg OTA, 21st day of study). Reducing the concentration of γ-globulins was significant compared to the control values, both immediately after the withdrawal of toxin from the feed, as well as after the period of feeding with no added toxin (42nd day of study) (Table 2, Figure 3).

Table 2. The influence of increasing concentrations ochratoxin A on broiler blood serum concentration of albumins α, β and γ-globulins [g/L]

Group	n	Concentrations, g/L (X±SD)		
		α-globulins	β-globulins	γ-globulins
		21st day		
Control	6	3.88 ± 1.00	3.84 ± 0.76	12.87 ± 1.88
0.5 mg/kg	6	4.29 ± 0.64	3.81 ± 0.65	12.66 ± 2.05
1.0 mg/kg	6	4.09 ± 0.47	4.05 ± 0.77	10.97 ± 1.79 b_1
1.5 mh/kg	6	3.75 ± 0.46	4.84 ± 0.58 * (p=0.0283); o (p=0.0159)	9.56 ± 0.85 ** (p=0.0031); oo (p=0.0065)
		42nd day		
Control	6	4.62 ± 0.49	5.26 ± 0.98	13.33 ± 1.84
0.5 mg/kg	4	2.77± 0.72 ** (p=0.0012)	5.21 ± 0.35	11.15 ± 1.26
1.0 mg/kg	4	4.50 ± 0.31 oo (p=0.0045)	4.87 ± 0.72	9.69 ± 1.27 ** (p=0.0091)
1.5 mg/kg	6	4.25 ± 0.79 o (p=0.0171)	4.93 ± 0.45	9.23 ± 1.40 ** (p=0.0015)

* – comparison with control group; o – comparison with 0. 5mg/kg group

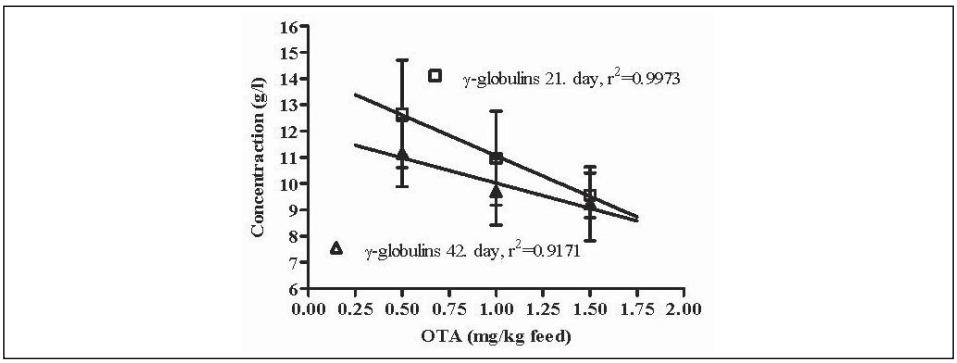

Figure 3. Effect of increasing concentrations of OTA (0.5, 1 and 1.5 mg/kg of feed) on the concentration of globulin fractions in the blood serum of treated broilers

DISCUSSION

The obtained results have demonstrated that the total concentration of blood serum proteins obtained in this investigation was between 32.46 ± 2.25 and 36.85 ± 1.12 g/L. Total protein concentration was mostly in agreement with the values described as physiological (Sirvydis *et al.* 2006; Stevanović *et al*. 1990). Similar results were reported by some authors (Kubena *et al*., 1985b; Kubena *et al.* 1989; Kubena *et al.,* 1994) although several authors (Ayed *et al.,* 1991; Singh *et al.,* 1990) reported somewhat higher values as normal proteinaemia in broilers. In the blood serum of experimental groups, mild differences influenced by OTA treatment were seen, but with no statistical significance. These findings did not confirm the results obtained by Kubena *et al.,* (1985b); Kubena *et al*., (1989); Kubena *et al.*, (1994) who suggested that the total blood serum protein concentration significantly decreased after treatment with OTA. On the other hand, the obtained results confirmed the findings of others authors (Huff and Ruff, 1981; Singh *et al*., 1990) who suggested that the total blood serum protein concentration was not significantly decreased after treatment with lower levels of dietary OTA.

Our results may be explained by the lower level of dietary OTA, as well as by the shorter duration of intoxication than in the Kubena studies (Kubena *et al*., 1985b; Kubena *et al.*, 1989; Kubena *et al*., 1994). The target organs for OTA toxicity in the animals are the kidneys with resulting failure to reabsorb the water and consequent increase of water consumption. Depending on the degree of kidney damage, water disbalance affected the concentrations of blood constituents. OTA has been reported to cause a dehydration-related increase of serum protein concentrations in pigs (Szczech *et al*., 1973), while the decrease of serum proteins without changes of A/G ratio may be connected with blood dilution. Thus, it could be concluded that the increasing dietary OTA levels 0.5, 1.0 and 1.5 mg/kg during one week did not have any negative effects on total blood serum proteins in broilers, which was in agreement with the similar conclusion reported by Chang *et al.* (1981). In addition, these results may support our previous findings from the trial using smaller doses of OTA (0.5 mg/kg) (Nedeljković Trailović *et al*., 2001). The serum albumin concentration in the control group (Table 1) was lower than the globulin concentration. Comparing the obtained results for albumins with normal values (Stevanović *et al*., 1990), it could be seen that they range close to the upper limit, which is in almost complete agreement with the findings of Kubena *et al.* (1985a; 1994) specifying concentrations of 11.3-14.2 g/L as normal albuminaemia. The same authors stated that the occurrence of low levels of OTA in the diets during the short period led to the decrease of serum albumins, while the obtained results in this trial were contrary. In the blood serum of treated birds, the dose-dependent increase of albumin concentration, in proportion to the dietary OTA level, was seen. During the restitution period (21st until 42nd day of the study), blood serum albumin concentration was significantly higher in all experimental groups compared with the control group of broilers (Table 2). These findings were in agreement with our previous report (Nedeljković-Trailović *et al*. 2001) although they did not confirm

the findings of some authors (Ayed *et al*., 1991; Kubena *et al.,* 1984; Kubena *et al.*, 1985a; Singh *et al*., 1990) obtained with higher doses of OTA (2 or more ppm). Increase of plasma albumin concentration was probably connected with the high affinity of OTA for plasma proteins. This could be an essential factor influencing toxicokinetics and toxicity of OTA (Kumagai, 1985; Uchiyama and Saito, 1987), but the relation between the high affinity of OTA for plasma proteins and toxicity was not yet entirely explained (Hagelberg *et al*., 1989). It was concluded that one of the primary reasons for albumin binding of OTA was to delay its elimination by limiting the transfer of OTA from the bloodstream to the hepatic and renal cells. The increased concentration of the plasma albumins could be interpreted as homeostatic mechanism. With the aim to keep OTA in the blood, the liver increased albumin production thus minimizing the tissue damage in target organs. Besides, increasing the albumin concentration, as part of a transport mechanism of nutrients, could be assumed to be a homeostatic mechanism by which the organism is attempting to secure enough carriers for the optimal transport of essential nutrients. This view is confirmed by the fact that according to our results after the period with no toxins in diet (21-42nd day) the concentrations of albumins in all treated groups of broilers were still higher than in the control, but the findings now are not dose-dependent.

Observing the concentration of total globulins and globulin fractions in the blood serum of chickens shows the opposite relationship. According to literature data (Bailey *et al*., 1990; Kubena *et al*., 1985a; Kubena *et al*., 1994) globulin concentration is higher than albumin concentration in the blood serum. Contrary, according to our results, seven days of treating with the toxin led to a dose-dependent decrease in the concentration of total serum globulins in treated broilers, but only the highest dose of toxin (1.5 mg/kg feed) showed significant effects.

It could be concluded that the low amounts of toxin (0.5 mg/kg feed) in the short period of time had no significant effects on globulin concentration, while its consumption in the higher amount (1.0 and 1.5 mg/kg feed) may result in the decrease of globulin concentrations, which is in accordance with the literature data (Kubena *et al*., 1985a; Kubena *et al*., 1985b; Kubena *et al.*, 1989; Kubena *et al*., 1994). One of the explanations for the decrease in globulin concentration could be the consequence of decreased reabsorption of proteins from the primary urine back to the blood or the inhibition of protein synthesis.

The obtained concentrations of α, β and γ-globulins in the blood serum of broilers in our study (Tables 2) were more or less in accordance with literature data (Valdivia *et al*., 2001; Sirvydis *et al*., 2006; Singh *et al*., 1990; Stevanović *et al*., 1990). Values of concentrations of α and β-globulins in the serum of treated broilers were mostly unchanged in comparison with the control, found during the entire period of examination. An exception is the reduced concentration of α-globulins 42nd day in broilers that were treated with 0.5 mg/kg and increased concentrations of β-globulins 21st day in broilers that received 1.5 mg/kg of toxin (Table 2). Therefore, these data confirmed that treatment with OTA did not have an important influence on α and β globulins. Opposite to the described, feeding the broilers with increasing doses of OTA had shown the dose-dependent reducing

effects on concentration of γ-globulins, which is clearly observable and even intensify during periods without toxins present in the food. The decreased concentrations of γ-globulins in the blood sera of broilers in all treated groups may confirm the immuno suppressive effect of OTA previously reported by Lea *et al.* (1989). Although there was no evidence to show that OTA inhibits the synthesis, particularly of IgM (Singh *et al*., 1990). It might have been that OTA decreased the synthesis of the immune globulins in the manner similar to aflatoxin. No matter which the exact mechanism was involved, it was obvious that the OTA was able to express the immunomodulatory effects earlier described by Hong *et al.* (1988) and later confirmed by Singh *et al.* (1990). Therefore, this also certainly confirms the dose-dependent effect of OTA on the changes of protein content in the serum of broilers.

The described differences in concentration and relations between sera protein fractions could be summarized in albumin/globulin ratio (A/G). The usage of low level dietary OTA resulted in a relatively mild decrease of albumin concentration. The use of feed with higher level of OTA increased the A/G ratio due to the increase of albumins and the decrease of γ-globulins. The toxicity of OTA is probably based on more than one direct and several indirect mechanisms. The primary effect was probably due to impaired protein synthesis caused by the competitive inhibition of the enzymes involved in the phenylalanine metabolism (phenylalanine tRNA synthetase) like a prerequisite for protein synthesis (Meisner and Meisner, 1981). Treatment with OTA in the diet exerted negative effects on γ-globulins concentration, especially when administered in the prolonged period. On the other hand, the duration of the treatment with OTA in our investigation did not probably last long enough to decrease the total protein synthesis. Thus, the total level of serum proteins was not changed under the influence of treatment with increasing OTA levels. The significant increase of albumins together with the decrease of the γ-globulin fraction was established. The A/G ratio suggested that globulins were the dominant protein fraction and the concentrations of α and β-globulins in the serum were within the physiological limits for broilers. It could be concluded that the increasing dietary OTA levels, in the range 0.5-1.5 mg/kg had possible cumulative effects on the blood serum proteins status in broilers. The observed effect is dose-dependent and directly or indirectly still exists after the cessation of toxin administration. Under natural circumstances in field cases, with the usual dietary OTA level between 0.2 and 1.0 mg/kg, the changes in total serum protein, as well as changes of protein fractions and A/G ratio could be only poor diagnostic tools for the ochratoxicosis in broilers. Nevertheless, these can be used as a valuable step in further investigations of toxic mechanism of OTA, especially on blood serum concentrations of proteins and their fractions.

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STATUS SERUMSKIH PROTEINA BROJLERA HRANJENIH OBROCIMA SA RASTUĆIM KONCENTRACIJAMA OHRATOKSINA A

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SADRŽAJ

Ispitivanje je trajalo 42 dan na ukupno 48 brojlera podeljenih u četiri jednake grupe. Preeksperimentalni period trajao je 14 dana, nakon čega su životinje tri

ogledne grupe tokom sedam dana hranjene obrocma koji su sadržali 0,5, 1,0 i 1,5 mg ohratoksina A (OTA) u 1kg hrane. Brojleri kontrolne grupe u toku eksperimentalnog perioda hranjeni su hranom bez dodatka toksina. Posle sedam dana tretmana uzimani su uzorci krvi od po šest životinja iz svake grupe (kontrolne i eksperimentalnih). Preostale životinje hranjene su obrocima bez toksina do 42. dana ogleda, nakon čega je krv uzorkovana i od njih. Koncentracija ukupnih proteina plazme bila je promenjena u zavisnosti od koncentracije OTA koji su brojleri dobijali hranom. Takođe je zabeleženo značajno i dozno-zavisno povećanje koncentracije albumina sa snižavanjem koncentracije gama-globulinske frakcije u serumu. Albulin/Globulin odnos (A/G odnos) ukazuje da su globulini bili dominantna frakcija proteina kod svih brojlera u ispitivanju. Koncentracija alfa i beta-globulina u serumu brojlera kretala se u fiziološkim granicama dok je koncentracija gama-globulina bila značajno snižena.

Može se zaključiti da rastuće koncentracije OTA u hrani brojlera ispoljavaju dozno-zavistan kumulativni efekt na status serumskih proteina. Ovaj efekat ostaje i posle prestanka dodavanja toksina u hranu.