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Experimental coccidiosis provoked by *Eimeria adenoeides* in turkey poults given ochratoxin A

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ABSTRACT

The progression of coccidiosis provoked by *E. adenoeides* was followed in turkey poults given ochratoxin A (OTA) in feed. More heavy and rapid progress of coccidiosis occurred in OTA-treated turkey poults than in those fed an OTA-free diet, as can be seen from the higher levels of oocyst and lesion indices and more rapid mortality in such birds. The concentration of total protein in serum was significantly increased in turkey poults infected with *E. adenoeides* and simultaneously given OTA. The serum concentration of uric acid was significantly increased in all experimental groups, especially in those exposed to OTA, and most notably in the group additionally infected with *E. adenoeides*. OTA induced degenerative changes in kidneys, liver and heart as well as depletion of lymphoid tissue in the lymphoid organs and a decrease in body mass. Coccidiosis also induced a growth depression in addition to caecal haemorrhages. Impairment of kidney function, histopathological changes and general growth depression were stronger when turkey poults infected with *E. adenoeides* were also given OTA.

Key words: mycotoxic nephropathy, ochratoxicosis, mycotoxins, ochratoxin A, coccidiosis, *E. adenoeides*

Introduction

Ochratoxin A is a widespread mycotoxin in animal feed and its significance for animal health has been comprehensively reviewed (KUIPER-GOODMAN and SCOT, 1989;

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MARQUARDT and FROHLICH, 1992). The major economic problems that have been reported when OTA-contaminated feed was fed in chicks were growth depression and increased mortality, although some other characteristic damage to many tissues have been also noted (HUFF et al., 1974; DWIVEDI and BURNS, 1984; DWIVEDI and BURNS, 1986; STOEV et al., 2000a; STOEV et al., 2002c). The main cause for increased mortality among chicks or pigs fed an OTA-contaminated diet appeared to be OTA-provoked immunosuppression (BURNS and DWIVEDI, 1986; STOEV et al., 2000a; STOEV et al., 2002c) resulting in higher susceptibility to secondary bacterial infections (STOEV et al., 2000b). Therefore, our observations on naturally encountered chick nephropathy in Bulgaria have identified a rather high incidence of mortality which can only partly be attributed to OTA-contaminated feed (STOEV et al., 2002b). Moreover, descriptions of pathology of spontaneous chick nephropathy vary considerably, especially if another secondary disease arises spontaneously along with OTA-induced nephropathy in chicks, which can often be seen because of the immunosuppression accompanying this nephropathy (STOEV et al., 2000a; STOEV et al., 2000b). Accordingly, it is important to clarify the complex clinicomorphological findings in such cases, which derive from both diseases, as well as to follow the progression of coccidiosis in the same cases. At present, there is very scarce and contradictory data on progression of coccidiosis in chicks fed on OTA-contaminated diet. For example, HUFF and RUFF (1982) have reported an increase of serum uric acid and an increase in relative mass of liver in chicks fed OTA and contaminated with *E. tenella*, but both the relative mass of liver and uric acid concentration in serum were decreased in chicks fed OTA and contaminated with *E. acervulina*. For that reason we decided to perform further investigations in this field in order to clarify these inconsistencies in the literature.

Materials and methods

OTA production. *Aspergillus ochraceus* isolate D2306, as used by TAPIA and SEAWRIGHT (1984) and STOEV et al. (2000b, 2002a) was grown on sterilized shredded wheat (40 g) in 500 mL conical flasks, moistened by a 40% (w/v) addition of sterile water and incubated on a rotary shaker at 27 ^{0}C for 2 weeks (HARRIS and MANTLE, 2001). The brown granular product, which bore no obvious sign of fungal growth or sporulation, was sterilized at 80 ^{0}C for 1 hour and stored at -20 ^{0}C. A sample was analyzed for ochratoxins. Batches typically contained OTA (about 2 mg/g) and a relatively small component of biologically-inactive alkaloid ochratoxin B (deschloro-ochratoxin A). No other mycotoxins were produced in this solid substrate fermentation process and the necessary dilution by approximately 10^{-3} when homogenized into chick ration produced only a minimal addition of other components of the moulded shredded wheat substrate.

Experimental design. Specific pathogen-free, one week-old turkey poults (breed BUT-9) were purchased and housed in wire floor cages with continuous infra-red lighting at a temperature suitable for their age. Commercial complete standard feed (Smesler, Stara Zagora, Bulgaria) with or without added OTA was available *ad libitum*. The birds were divided into 3 experimental groups and 1 control group (10 birds in each group) and treated with either or both OTA and *E. adenoeides*, as presented in Table 1.

Table 1. Experimental design: feed levels of ochratoxin A and inoculation with *E. adenoeides* (at the beginning of the experiment) of various experimental groups of turkey poults.

Group	OTA in feed (ppm - mg/kg)	Inoculation with *Eimeria adenoeides* (4×10^5 oocysts per turkey-poult *per os*)
I	none	*E. adenoeides*
II	2	none
III	2	*E. adenoeides*
Control	none	none

Measurements. Turkey poults were weighed at the beginning of the experiment and again after one week. In order to follow pathomorphological changes in various internal organs, as well as oocyst and lesion indices, birds from each group were slaughtered one week after the beginning of the experiment (one week after infection with *E. adenoeides*), as the first mortalities occurred in Group III during that time (Table 1).

Histological examination. Tissues for histological examination were taken from kidneys, liver, lung, heart, thymus, bursa of Fabricius, spleen, intestine, cerebellum, brain and medulla and fixed in 10% neutral buffered formalin. The fixed tissues were processed for paraffin embedding, sectioned at 6 μm and stained with haematoxylin-eosin.

Parasitological examination. The required quantity of oocysts was obtained from naturally susceptible turkey poults (aged two weeks) and inoculated experimentally with oocysts of *E. adenoeides* according to LOZANOV (1980). The oocysts were preserved in 2.5% solution of potassium bichromate ($K_2Cr_4O_7$) at 4 °C until used for inoculation (Table 1). The oocyst and lesion indices in slaughtered turkey poults were examined according to CUCLER (1959) and as modified by JOHNSON and REID (1970), respectively (Table 2).

Haematological and biochemical examination. Blood samples were taken at slaughter at the end of the 7th day of the experiment and examined for various biochemical parameters within 1-2 h of collection, immediately after separation of serum. Serum total protein was measured by Bio-La-Test (Lachema Diagnostica, Brno, Czech Republic)

and uric acid by EnzUric-FT-test (Labordiagnostica, Gopecke, Germany). The serum enzyme activity of ASAT (asparate-aminotransferase) was measured by Cormay test (Smolenskiego, Warsaw, Poland).

Table 2. Mean values of lesion and oocyst indices in slaughtered turkey poults (7 days after inoculation with *E. adenoeides*).

Group	N° examined chicks	Lesion index#	Oocyst index##
I *(E.ad.)*	10	$1.90^{a} \pm 0.23$	$11.81^{a} \pm 1.21$
II *(OTA)*	10	-	-
III (OTA+*E.ad.*)	8	3.05 ± 0.20	27.00 ± 3.66
Control	10	-	-

± SEM (standard error of the mean)
a - significant difference compared to group III ($P<0.05$);
- Lesion index (the maximum is 4) was examined using the following scheme:
Assessment 1 - scarce petechial haemorrhages on the mucosal surface and slight thickening of intestinal mucosa
Assessment 2 - a small number of haemorrhages up to pinhead size on the mucosal surface, oedema and thickening of the intestinal mucosa
Assessment 3 - many haemorrhages up to pinhead size on the mucosal surface, oedema and thickening of the intestinal mucosa, degenerative changes in the mucosal epithelium, caecums contain necrotic cheese-like debris
Assessment 4 - many haemorrhages up to pinhead size on the mucosal surface, oedema and pronounced thickening of the intestinal mucosa, strong degenerative changes in the mucosal epithelium, caecums are full of necrotic cheese-like debris containing many oocysts and blood traces
- Oocyst index (maximum 40) was examined using the following scheme:
Assessment 0 - from 0 to 0.1 million oocysts in the intestine of chicks
Assessment 1 - from 0.1 to 1 million oocysts
Assessment 10 - from 1 to 5 millions oocysts
Assessment 20 - from 5 to 10 millions oocysts
Assessment 40 - more than 10 millions oocysts

Statistical methods. Non-parametric Mann-Whitney in addition to Student's t-test was used to estimate significant differences between the mean values of various parameters in different groups of turkey poults.

Results

Clinical observation. Diarrhoea was the main clinical sign seen first in turkey poults given OTA (Groups II and III) on day 3 from the beginning of the experiment, but the same was also seen in turkey poults of Group I, 5 days after inoculation with *E. adenoeides*, and was most pronounced in turkey poults infected with *E. adenoeides* and

simultaneously given OTA (Group III) at that time. In addition, there were blood traces in the watery diarrhoeic contents in the turkey poults of the last group. Other clinical signs, depression, weakness and dullness, goose plumage, reduction of feed intake and growth depression, occurred in all experimental groups, although mainly in Groups II and III. Two turkey poults in Group III died at day 6 of the experiment, due to the earlier and heavy progression of coccidiosis, as can be seen from the higher values of lesion and oocyst indices (Table 2) and the significant decrease in body mass (Table 3) in slaughtered turkey poults of the same OTA-treated group.

Body mass. A statistically significant decrease in body mass was observed in all experimental groups given OTA and/or infected with *E. adenoeides*, compared to the control group, but that decrease was most marked in the group given OTA and simultaneously infected with *E. adenoeides* (Table 3).

Table 3. Mean values of body mass (b.m.) in turkey-poults of various experimental groups at the beginning and at end of the first week of the experiment.

Group	1st day b.m. (g)	1st day N° of turkey-poults	7th day b.m. (g)	7th day N° of turkey-poults
I *(E.ad.)*	130.5 ± 4.1	10	189.0*a ± 7.3	10
II *(OTA)*	127.5 ± 5.7	10	187.0*a ± 8.9	10
III *(OTA+E.ad.)*	128.0 ± 4.2	10	153.0* ± 7.1	8
Control	135.5 ± 5.4	10	215.0a ± 9.2	10

± SEM (standard error of the mean)
* - significant difference compared to controls (P<0.05)
a - significant difference compared to group III (P<0.05)

Biochemical and haematological findings. Total protein in serum was significantly increased only in turkey poults treated with OTA and *E. adenoeides* simultaneously (Table 4).

A significant increase was seen in serum uric acid in all experimental groups, but most notably in OTA-treated groups. That increase was highest in turkey poults infected with *E. adenoeides* and simultaneously given OTA (Table 4).

There were no significant changes in serum enzyme activity of ASAT in all experimental groups compared to the control (Table 4).

Gross pathology and parasitological findings. There were very few subcutaneous haemorrhages in OTA-treated turkey poults in Groups II and III. Kidneys and liver in these birds were congested and enlarged and their gall bladder was also distended with bile. The mucosal surface of small intestine was hyperaemic and covered with mucous fluid. Meninges were slightly hyperaemic.

Table 4. Mean serum values of total protein, ASAT (asparate-aminotransferase) and uric acid in turkey-poults of various experimental groups at the end of the first week of the experiment.

Group	Total protein (g/L)	ASAT (U/L)	Uric acid (μmol/L)	Number of turkey-poults
I *(E.ad.)*	48.30 ± 0.76	89.9 ± 2.2	74.8*[a] ± 11.3	10
II *(OTA)*	45.40 ± 2.73	85.8 ± 1.8	543.8* ± 81.3	10
III *(OTA+E.ad.)*	58.91* ± 2.22	85.5 ± 6.1	750.4* ± 97.2	8
Control	46.30 ± 1.79	86.6 ± 3.4	27.9[a] ± 4.4	10

± SEM (standard error of the mean)
* - significant difference compared to controls (P<0.05)
[a] - significant difference compared to group III (P<0.05)

In turkey poults infected with *E. adenoeides* (Groups I and III) there were many haemorrhages up to 2-3 mm in size on the mucosal surface of the caecums, and in some cases of the ileum. A pronounced thickening of the intestinal mucosa of the caecums, ileum and colon was also seen. Caecums were full of necrotic cheese-like debris,

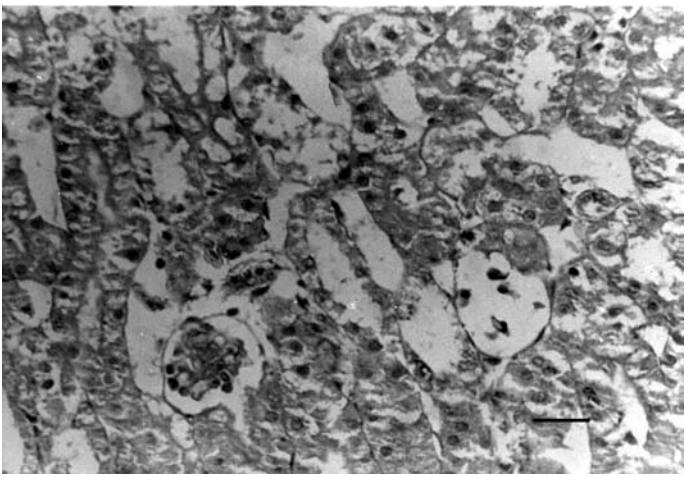

Fig. 1. Photomicrograph of kidney in turkey poult fed 2 ppm OTA in diet and infected with *E. adenoeides*. Granular degeneration in epithelial cells of the tubules. H&E; ×300; scale bar = 33 μm.

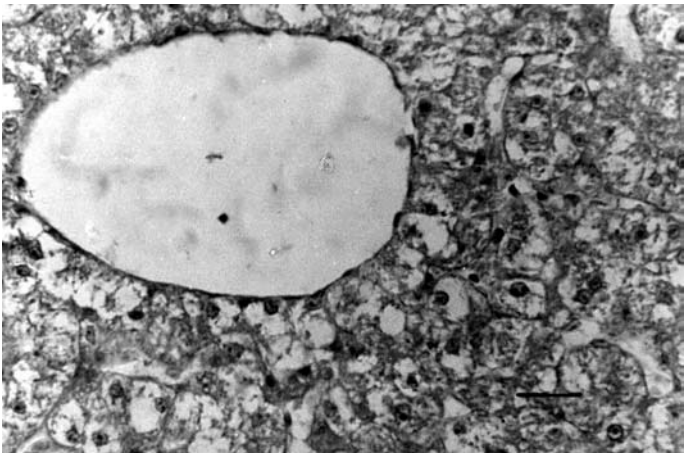

Fig. 2. Photomicrograph of liver in turkey poult fed 2 ppm OTA in diet and infected with *E. adenoeides.* Granular and slight vacuolar degeneration in hepatocites. H&E; ×300; scale bar = 33 μm.

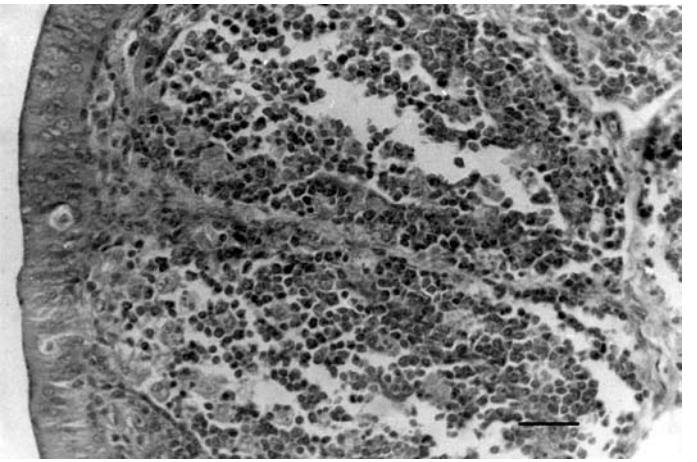

Fig. 3. Photomicrograph of bursa of Fabricius in turkey poult fed 2 ppm OTA in diet and infected with *E. adenoeides.* Depletion of lymphoid cells in the central part of the lymph follicles. H&E; ×200; scale bar = 50 μm.

containing some blood traces, especially in turkey poults given OTA and *E. adenoeides* simultaneously. At the same time, the content of the small intestine and colon was watery, and the mucosal surface was covered with mucous fluid.

The number of oocysts in the intestine and the values of the oocyst indices were also clearly higher in turkey poults given OTA in diet (Table 2).

Histopathology. No histopathological changes were found in internal organs of the controls.

In kidneys of OTA-treated turkey poults, peritubular capillaries were hyperaemic. Degenerative changes (mainly granular degeneration) in epithelial cells of the proximal convoluted tubules were seen (Fig. 1) and focal mononuclear cell infiltration was observed in the renal interstitium. Some tubules contained necrotic debris in the lumen. Limited proliferation of connective tissue and activation of capillary endothelium were also noticed in the same turkey poults. Such damage was more pronounced in turkey poults given OTA and *E. adenoeides* (Group III) than in those given OTA alone (Group II). In turkey poults treated with *E. adenoeides* alone (Group I) only slight hyperaemia of peritubular capillaries and slight granular degeneration in epithelial cells of the proximal convoluted tubules were seen.

Granular or vacuolar degeneration was seen in the livers of OTA-treated groups, but this was most marked in Group III, also infected by *E. adenoeides* (Fig. 2). Also, there was activation of capillary endothelium and Kupffer's cells, hyperaemia and pericapillary oedema, as well as perivascular infiltration of mononuclear cells in the same turkey poults. Only slight hyperaemia of capillaries and granular degeneration in hepatocytes were seen in Group I turkey poults, treated with *E. adenoeides* alone.

In lymphoid organs, degenerative changes were seen only in turkey poults in Groups II and III. There was a strong depletion of lymphoid cells in the cortical zone of the thymus - the region between medulla and cortex was not well defined or the cortex became very thin. Depletion of lymphoid cells (Fig. 3), interfollicular oedema (Fig. 4) and degenerative changes (karyopyknosis and karyorrhexis) in the lymph follicles were seen in the bursa of Fabricius. A slight cellular depletion or degenerative changes were also observed in the white pulp of germinal centres in the spleen of turkey poults in the same groups. No histopathological changes were seen in the lymphoid organs in Group I turkey poults.

Degenerative and slight necrotic changes, as well as a desquamation of surface- or glandular- mucosal epithelium of duodenum and jejunum, were seen in Group II and III turkey poults.

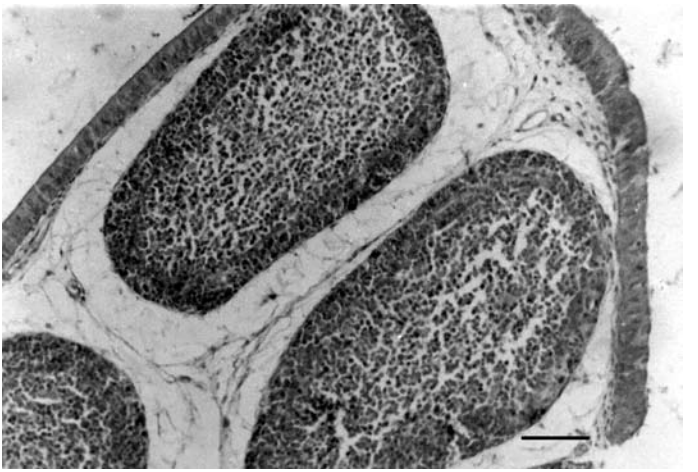

Fig. 4. Photomicrograph of bursa of Fabricius in turkey poult fed 2 ppm OTA in diet. Interfollicular oedema and slight depletion of lymphoid cells in the central part of the lymph follicles. H&E; ×100; scale bar = 100 μm.

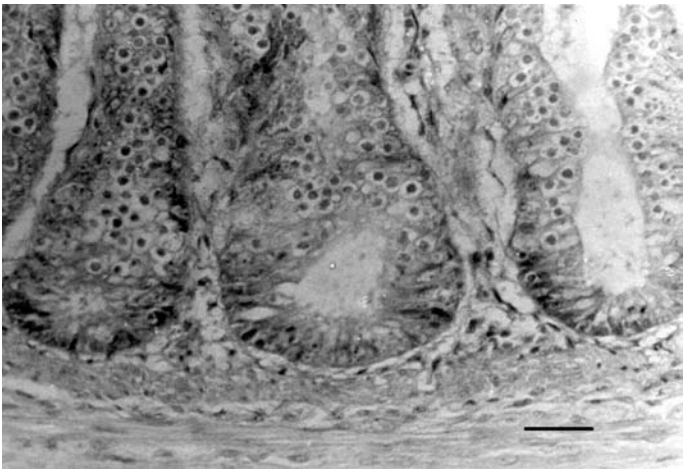

Fig. 5. Photomicrograph of caecal mucosa in turkey poult fed 2 ppm OTA in diet and infected with *E. adenoeides.* Many oocysts or schizonts in the glandular epithelium. H&E; ×200; scale bar = 50 μm.

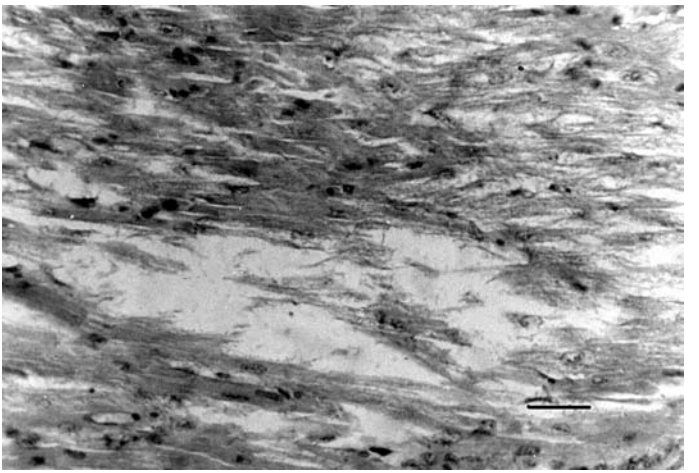

Fig. 6. Photomicrograph of heart of turkey poult fed 2 ppm OTA in diet and infected with *E. adenoeides.* Slight granular degeneration and lytic changes of myofibrils. H&E; ×200; scale bar = 50 µm.

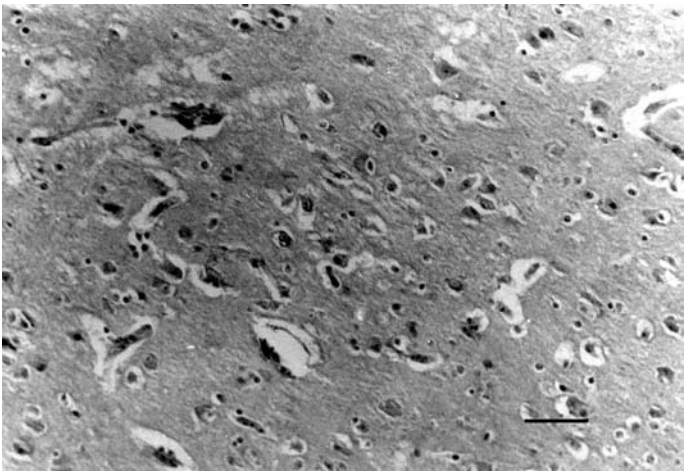

Fig. 7. Photomicrograph of brain in turkey poult fed 2 ppm OTA in diet and infected with *E. adenoeides.* Pericellular and pericapillary oedema. H&E; ×200; scale bar = 50 µm.

Small haemorrhages, hyperaemia of vessels, oedema in the submucosa, slight mononuclear infiltration and siderocytes in the lamina propria, as well as a large number of oocysts or schizonts in the surface and glandular epithelium and caecal content were seen in the caecums in turkey poults infected with *E. adenoeides* (Groups I and III), most notably in the latter group (Fig. 5). Strong degenerative changes and desquamation of surface or glandular epithelium were also present in the mucosa of caecums in chicks infected with *E. adenoeides*. Also, there was necrotic debris and many oocysts in caecal content in the same chicks.

A perivascular oedema and irregular staining, as a result of the increased eosinophilia of some myofibrils, were seen in the heart of turkey poults of OTA-treated groups. Slight granular degeneration and lytic changes of myofibrils (Fig. 6) and activation of capillary endothelium were also observed in these turkey poults. In turkey poults treated with *E. adenoeides* only (Group I), hyperaemia of vessels and granular degeneration of myofibrils were seen, albeit in small proportions.

In the lung, only perivascular or peribronchial mononuclear cell infiltration and slight oedematous changes were noted in turkey poults of OTA-treated groups.

Pericellular or pericapillary oedema in the brain (Fig. 7) was also noted in turkey poults in OTA-treated groups. Additionally, slight lytic changes in neurons and glia cells of the brain in the same chicks were evident. In the cerebellum of these turkey poults, oedema and slight degenerative changes were observed in the region of Purkinje's cells, although rarely in molecular or granular layers. In addition, slight oedematous changes in the white substance of the cerebellum were occasionally seen in turkey poults in Groups II and III. Slight lytic changes in neurons were also noted in the lumbosacral region of the medulla in some turkey poults of the same groups. Only slight hyperaemia of capillaries and a few lytic changes in neurons and glia cells were seen in the brain and the molecular or granular layers of the cerebellum of Group I turkey poults treated with *E. adenoeides* alone.

Discussion

Intensity of clinical signs, macroscopic and histopathological changes, growth depression, as well as impairment of kidney function, expressed by serum levels of uric acid, were stronger when turkey poults infected with *E. adenoeides* were also given OTA. Moreover, coccidiosis progresses strongly and rapidly in OTA-treated turkey poults, as can be seen from the changes in lesion and oocyst indices, and especially from early chick mortality. This could be explained by OTA-provoked immunosuppression and increased sensitivity to various infectious or parasitic diseases (STOEV et al., 2000a; STOEV et al., 2000b).

Growth depression in OTA-treated chicks, according to MOHIUDDIN et al. (1993), may be due to the impairment of protein synthesis by OTA.

Histopathological changes in kidneys of OTA-treated turkey poults corresponded well with the increase in serum concentration of uric acid, which showed impairment in kidney function. Degenerative changes in the epithelial cells of kidneys and liver probably are due to the route of elimination of OTA via kidneys, and partly via liver, due to enterohepatic recirculation and hepatobiliary manner of excretion of OTA (FUCHS, 1988), exerting a direct toxic effect of OTA on these organs (DWIVEDI and BURNS, 1984; STOEV et al., 2000a). The slight granular degeneration in hepatocytes in turkey poults treated with *E. adenoeides* alone could be due to the resorption of toxic products as a consequence of decomposition of necrotic epithelial debris in the intestinal content (LOZANOV, 1983; LOZANOV and KOYNARSKY, 1985).

Degenerative changes and depletion of lymphoid cells in the lymphoid organs in OTA-treated turkey poults may accord well with immunosuppressive effects that OTA can exert in contamination levels 2-4 ppm, as reported by some authors (HOLMBERG et al., 1988; HARVEY et al., 1992; KOZACZYNSKI, 1994). Therefore, heavy progression of some infectious (STOEV et al., 2000a; STOEV et al., 2000b) and even parasitic diseases could be expected, as was demonstrated in the present experiment. The OTA-provoked immunosuppression could be explained by the inhibition of protein synthesis and the subsequent delay in cell division in the immune system (HARVEY et al., 1992). The impaired protein synthesis in lymphocytes could lead to impairment in their activation, differentiation and proliferation. In addition, the decreased phagocytic activity of natural killer cells and T-killer cells, which is probably due to a decrease in basal interferon (HARVEY et al., 1992), may additionally deteriorate the immune response of OTA-treated chicks. In the present experiment it was demonstrated in practice how the known OTA suppression of humoral and cellular immunity (GRY et al., 1991; STOEV et al., 2000a; STOEV et al., 2000b) can exacerbate the progression of coccidiosis provoked by *E. adenoeides*, and can also lead to mortality among turkey poults at day 6 of the infection.

The perivascular oedema seen in various internal organs, in addition to some muscular haemorrhages, might be due to vascular damage provoked by OTA (STOEV et al., 2000a; STOEV et al., 2002c). Furthermore, a disturbance of blood clotting due to a reduction in the concentration of fibrinogen in the blood and an increase in the prothrombin time, observed as a response of ochratoxicosis (DOERR et al., 1981; PRIOR and SISODIA, 1978), could also contribute to these muscular haemorrhages. However, haemorrhages in the caecums and ileum are commonly observed, because of localization of *E. adenoeides* in this site (LOZANOV and KOYNARSKY, 1985).

It can be supposed that the increase in the serum total protein only in turkey poults treated with *E. adenoeides* and OTA simultaneously could be due to the complicated

clinicomorphological findings in these cases, and especially to the strong diarrhoea and dehydration of the same chicks.

The marked increase in the serum level of uric acid in both groups treated with OTA showed that the function of kidneys was significantly impaired, which is in agreement with our previous investigations on OTA-toxicity (STOEV et al., 1999; STOEV et al., 2000a; STOEV et al., 2002b, STOEV et al., 2002c). However, the results of the present study somewhat contradicts the conclusion made by HUFF and RUFF (1982), in that the serum concentration of uric acid decreased in chicks fed OTA and which simultaneously developed coccidiosis, as was reported in the same paper. This contradiction is probably due to duodenal localization of *Eimeria acervulina*, used in the same study. It is known that OTA-absorption is realized in the duodenal and jejunal part of the intestine, and localization of *E. acervulina* in the same part of the intestine could worsen OTA-absorption. This could lead to slight degenerative changes in kidneys, followed by lower levels of uric acid in such cases. However, the localization of *E. adenoeides* in our case is in the caecal part of the intestine, which could not worsen OTA-absorption. Moreover, resorption of certain toxic products from the caecum as a consequence of strong degenerative change in caecal wall, and decomposition of necrotic epithelial debris in intestinal content, could increase degenerative changes in kidneys. That could explain the higher levels of serum uric acid in turkey poults simultaneously treated with OTA and *E. adenoeides* compared to turkey poults treated with OTA only, as was seen in our case.

Concerning the serum enzyme activity of ASAT, it can be concluded that this parameter, surprisingly, cannot be changed in any of the OTA-treated chicks or turkey poults, which accords well with our previous investigations on serum enzyme activity in chicks fed OTA-contaminated diet (STOEV et al., 1999).

In conlcusion, the present experiment demonstrated in practice that certain often encountered parasitic diseases, such as coccidiosis provoked by *E. adenoeides*, can progress more strongly and rapidly in OTA-treated turkey poults, and can also lead to earlier mortality. The clinicomorphological findings in such cases may be complicated, because mortality derived from both diseases.

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KOYNARSKY, **V.**, **S. D. STOEV**, **N. GROZEVA**, **T. MIRTCHEVA**: **Pokusna kokcidioza uzrokovana vrstom *Eimeria adenoeides* u purića koji su dobivali ohratoksin A. Vet. arhiv 77, 113-128, 2007.**

SAŽETAK

Istraživan je tijek kokcidioze uzrokovan protozoonom *E. adenoeides* u purića koji su dobivali ohratoksin u hrani. Znatno teži oblik bolesti uočen je u purića pokusne u odnosu na kontrolnu skupinu. Razlika je uočena na razini broja oocista, stupnja oštećenja i bržeg uginuća. Koncentracija ukupnih proteina u serumu bila je također značajno povišena u pokusne skupine. Koncentracija mokraćne kiseline u serumu bila je osobito povišena u

skupini invadiranoj kokcidijama i hranjenoj hranom s primiješanim ohratoksinom. Ohratoksin je uzrokovao degenerativne promjene u bubrezima, jetrima i srcu kao i depleciju limfoidnog tkiva te smanjenje tjelesne težine. Kokcidioza je također uzrokovala smanjeni rast, ali i krvarenje u slijepim crijevima. Patohistološke promjene, smanjena funkcija bubrega kao i opće smanjen prirast bili su izraženiji u invadirane skupine koja je u hrani dobivala ohratoksin.

Ključne riječi: nefropatija, ohratoksikoza, mikotoksini, ohratoksin A, kokcidioza, *Eimeria adenoeides*