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Original article

Spontaneous mycotoxic nephropathy in Bulgarian chickens with unclarified mycotoxin aetiology

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Abstract – Histopathological, biochemical and toxicological investigations of tissues and blood of normally slaughtered chickens exhibiting different frequencies (1–2%, 40–50% and above 80%) of nephropathy changes (congested or pale and enlarged kidneys) at the slaughtering meat inspection were carried out to elucidate the aetiology of nephropathies of chickens encountered in Bulgaria. A close relationship was observed between the frequency of this nephropathy and the rate of nephrotoxic mycotoxin ochratoxin A in muscles, kidneys and livers of chickens, but the levels of ochratoxin A in corresponding feed samples (0.1–0.3 ppm) were significantly lower than the levels (2–4 ppm) required to reproduce such nephropathy. Clinicomorphological changes such as nervous symptoms, vascular and oedematous changes in various internal organs and the brain, and subcutaneous or liver and kidney haemorrhages in addition to known degenerative changes in the kidneys, liver and lymphoid organs differed from the classical description of the nephropathy made in Scandinavia. The conclusion is that the Bulgarian chicken nephropathy may have a multitoxic aetiology because it cannot be explained by the concentration of ochratoxin A alone.

mycotoxic nephropathy / ochratoxicosis / mycotoxin / ochratoxin A / pathology

Résumé – Néphropathie mycotoxique spontanée sans étiologie mycotoxique déterminée chez des poulets bulgares. Des études histopathologiques, biochimiques et toxicologiques des tissus et du sang de poulets montrant des fréquences variées (1–2 %, 40–50 % et supérieures à 80 %) de changements néphropathiques (reins congestionnés ou pâles et hypertrophiés) lors de l'inspection des viandes à l'abattoir, ont été entreprises afin de déterminer l'étiologie des néphropathies du poulet en Bulgarie. Une relation étroite a été observée entre la fréquence de cette néphropathie et le taux de la

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mycotoxine ochratoxine A néphrotoxique dans le muscle, le rein et le foie de poulet. Cependant, les taux d'ochratoxine A dans les échantillons de nourriture correspondants (1,1–0,3 ppm) étaient significativement inférieurs aux taux requis pour obtenir une telle néphropathie (2–4 ppm). Les changements clinico-morphologiques différaient de la description classique de cette néphropathie faite en Scandinavie par les symptômes nerveux, les changements vasculaires et œdémateux dans divers organes internes et dans le cerveau, et par les hémorragies sous-cutanées et du foie et des reins, qui venaient s'ajouter aux changements dégénérescents déjà connus dans les reins, le foie et les organes lymphoïdes. En conclusion, cette néphropathie des poulets bulgares pourrait avoir une étiologie multitoxique, car elle ne peut pas être expliquée uniquement par la concentration d'ochratoxine A.

néphropathie mycotoxique / ochratoxicose / mycotoxine / ochratoxine A / pathologie

1. INTRODUCTION

Mycotoxic nephropathy in chickens (MNC) has been comprehensively reviewed [3, 15, 16, 20]. The cause is nephrotoxic mycotoxins, predominantly ochratoxin A (OTA), present in chick feed which has been stored under conditions of excessive moisture. While agreeing that the most important toxicological target of OTA in the pig is the kidney, the principal descriptions of the pathology of MNC vary considerably. Moreover, the clinico-morphological picture of spontaneous MNC in Bulgaria differs significantly from the classical description of the nephropathy made in Scandinavia [5] and is also encountered in the same regions of the country where a mycotoxic porcine nephropathy is evident, which supposes the same multicausal nature [26, 27]. Therefore, it was necessary to elucidate the aetiology of this nephropathy and to describe in full its clinicomorphological picture.

2. MATERIALS AND METHODS

2.1. Animals

Chickens (four line broiler hybrid Cornish × Plymouth Rock) from 5 large-scale chicken farms with known prevalence of MNC as well as chickens from farms without nephropathy were subject to biochemical, histological, ultrastructural and toxicological examinations.

2.2. Biochemical examination

Blood for serum examination was taken from the wing vein of chickens, originating from batches with 40–50% or above 80% nephropathies in each of the affected farms as well as from chickens originating from healthy farms (10 chickens from each farm) during 1997 and examined for various biochemical parameters immediately after separation of the serum (within 1–2 h of collection). Cholesterol was measured by the test of Boehringer Mannheim (Mannheim, Germany); total protein by the Bio-La-Test (Lachema Diagnostica, Brno, Czech Republic); and uric acid by the EnzUric-FT-test (Labordiagnostica, Gopecke, Germany).

2.3. Histological examination

Materials for histological examination were taken from 100 chickens with MNC-changes in kidneys (congested or pale and enlarged kidneys), recognized during meat inspection. Ten chickens from each of the affected farms were examined during 1997 and 1998. Samples were also taken from 10 chickens without nephropathy, originating from healthy farm. Samples from the kidneys, liver, heart, thymus, bursa Fabricii, spleen, intestine, cerebellum, brain, medulla and bone marrow were fixed in 10% neutral buffered formalin or processed for freezing microtome. The freezing materials were stained with Sudan III to identify the fat. The fixed tissues were processed for paraffin embedding, sectioned

at 6 μm and stained with haematoxylin-eosin. Periodic acid- Schiff (PAS) stain was also used to identify lipoprotein, glycoprotein or mucoprotein substances in various tissues and cell components, and especially to show the thickening of basement tubular membranes (with lipoprotein structure). Some materials were stained according to Weigert iron haematoxylin to show the presence or absence of fibrin in various cyst formations.

2.4. Ultrastructural examination

Part of the materials were prefixed in 5% glutaraldehyde for 2 h. The glutaraldehyde fixed tissues were washed in phosphate buffer (pH 7.4) for 2 h, fixed in 2% osmium tetroxide for 1 h and embedded in "Durcupan" (Fluka Chemie AG CH 9470, Switzerland). The sections were stained with uranyl acetate and lead citrate and examined with an Opton 10C electron microscope (Opton, Oberkohen, Germany).

2.5. Toxicological examination

Randomly selected tissue samples were taken at slaughter time from the kidneys and livers of chickens originating from batches with about 40–50% nephropathy on 2 of the affected farms (4 chickens from each farm) and from kidneys and livers of 4 chickens without nephropathy, originating from healthy farm during 1997. Also, randomly selected serum samples were taken from the same batches of chickens (5 chickens from each batch) with and without nephropathy. In order to establish a relationship between the frequency of the nephropathy and the contamination levels of ochratoxin A (OTA), randomly selected muscle samples were taken at slaughter time from three batches of chickens (15 chickens from each batch) with different prevalences of nephropathy: 1–2%, 40–50% and above 80% during the spring of 1997. All samples were frozen at –20 °C until toxicological examination. The extraction and purification procedures of serum were performed according to a modified method of Beker and Radic [2], but those of tissue samples were according to Bauer et al. [1]. After that, the samples were analysed for OTA by HPLC (high performance liquid chromatography) with a detection limit of 0.2 ng OTA/mL. Feed samples from commercially prepared broiler-complete feed were also collected from the same 5 affected farms (1 sample from each farm – a total of 5) with 40-50% or above 80% nephropathies and were analysed for OTA according to Hald et al. [11]. Five feed samples from farms without nephropathy (during a long period of time) were also analysed by the same procedure.

2.6. Statistical methods

Non-parametric Mann-Whitney in addition to Student t-test were used to estimate significant differences between the mean values of various parameters in different groups of chickens.

3. RESULTS

3.1. General observations

The occurrence of nephropathy in different batches of slaughtered chickens varied from 1–2% up to 90–100%. The continuance of this nephropathy, also differed widely from one month to about 5–6 months or even throughout a year. The nephropathy in chickens was usually observed during the spring and summer, similarly to porcine nephropathy and in the same regions as well. The problem seemed to come from certain feed plants whose grain had not been properly dried after harvest. A loss of body weight frequently accompanied this nephropathy, but after changing the feed, the problems with poor growth of chickens disappeared. The

frequency and duration of the nephropathy in different batches of slaughtered chickens varied significantly and depended on the duration of feeding of various suspected feeds stored in poor conditions and at high humidity.

3.2. Clinical observations

The main clinical signs, observed mainly in chickens of batches from farms with high frequency of nephropathy, were the following: weakness and dullness, growth depression, some nervous symptoms such as: torticollis, emprosthotonus, lurch, reeling gait (staggering step), tremor, side-split, sitting posture or layer fashion with tense or flexion of legs.

3.3. Clinical biochemistry

Serum levels of total protein and cholesterol were significantly ($p < 0.05$) decreased, whereas serum levels of uric acid were significantly ($p < 0.05$) increased in all batches with high frequency of nephropathy compared to batch without nephropathy (Tab. I).

3.4. Gross pathology

Macroscopic examination revealed a few muscular haemorrhages and subcutaneous oedema in some chickens with well manifested nephropathy. The kidneys and livers in cases with MNC were congested and enlarged, often with haemorrhages, but in some cases the kidneys were pale. The gall bladder was often enlarged and filled with bile. The mucosal surface of the duodenum and jejunum was hyperaemic and covered with mucoid fluid, whereas the lymphoid organs (thymus, bursa of Fabricius and spleen) were obviously diminished in size. The main part of the bone marrow had a grey-white colour, whereas the normal red colour was preserved only in a few places. The meninges were slightly hyperaemic.

3.5. Histopathology

Pathomorphological investigation of kidneys revealed degenerative changes in epithelial cells of the proximal convoluted tubules (Fig. 1) as well as a nodular infiltration of mononuclear cells and slight proliferation of connective tissue in the renal interstitium. Hyperaemia of peritubular capillaries in the kidneys of chickens with manifested macroscopic changes such as enlargement and congestion was seen. In this case, when kidneys were enlarged but pale, the microscopic picture was dominated by proliferation of connective tissue and degenerative changes in the proximal epithelium, but the hyperaemic changes were slight. Oedema, activation of capillary endothelium, hypercellularity of glomerules, tubular atrophy and thickening of basement tubular membranes in the kidneys were also seen in the last case. In the liver, cloudy swelling, granular degeneration and rarely

Table I. Mean (±SEM, $n = 10$) serum values of total protein, uric acid and cholesterol in chickens from different farms with high frequency or without nephropathy.

% of nephropathy in studied farms	Total protein (g/L)	Uric acid (μmol/L)	Cholesterol (mmol/L)
0	30.6 ± 0.66	167.1 ± 8.6	5.25 ± 0.12
40–50	25.1 ± 1.23	356.2 ± 34.9	3.72 ± 0.12
40–50	24.9 ± 0.77	431.1 ± 37.0	3.69 ± 0.09
above 80	22.5 ± 0.64	458.5 ± 35.8	3.38 ± 0.11
above 80	23.5 ± 0.67	450.6 ± 40.2	3.10 ± 0.11
above 80	21.8 ± 0.57	477.3 ± 24.6	3.04 ± 0.09

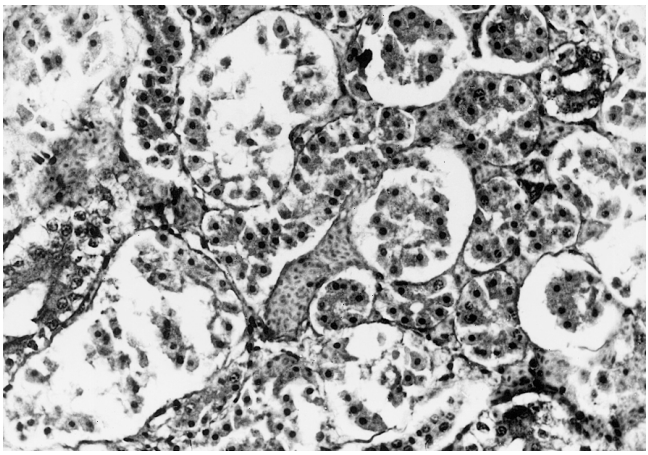

Figure 1. Photomicrograph of kidney in chicken with spontaneous mycotoxic nephropathy. Strong degenerative changes in epithelial cells of the proximal convoluted tubules. H/E. 260×.

fatty changes of the hepatic cells were observed. Activation of Kupffer cells, hyperaemia and pericapillary oedema as well as perivascular mononuclear cell infiltration were also noticed.

Pathomorphological investigation of lymphoid organs revealed strong degenerative changes in the lymphoid tissue. A depletion of lymphoid cells, interfollicular oedema and pronounced degenerative changes (karyopyknosis and karyorrhexis) as well as some necroses and cyst formations in the lymph follicles of bursa Fabricii (Fig. 2) were found. The thymus showed degenerative changes and a depletion of lymphoid cells in the cortical zone as well as congestion and haemorrhages. Cellular depletion and slight degenerative changes were found in the white pulp of germinal centres in the spleen. These centres were also reduced both in number and in size.

Similar degenerative changes and depletion of lymphoid cells were also seen in the lymph follicles of intestinal mucosa. A few little foci of necrosis and loss of surface epithelium as well as degenerative changes in the glandular epithelium were noticed in the intestinal mucosa.

In the heart, perivascular oedema, lytic changes and irregular staining as a result of the increased eosinophilia of some myofibrils, were noticed. Granular degeneration and intermuscular mononuclear cell infiltration were rarely observed. A perivascular, peribronchial or interalveolar mono- nuclear cell infiltration and hyperplasia or proliferation of alveolar epithelium were only seen in the lungs.

In the brain, a pericellular or pericapillary oedema and lytic changes in ganglionic and glia cells (Fig. 3) were seen in all chickens with pronounced nephropathy. The same changes were especially well manifested in the lobus opticus of the brain, where macroscopically visible small cysts were recognized in three cases. In the cerebellum, perivascular and peri- cellular oedema as well as slight degenerative changes in the region of the Purkinje cells and rarely in molecular and granular layers were observed. Oedematous changes were often found in the white substance of the cerebellum. In the lumbosacral region of the medulla, lysis and sclerosis in neurons were mainly seen.

In the bone marrow, cell depletion and fatty changes were only noticed.

Histopathological changes in internal organs were not seen in chickens from batches without nephropathy.

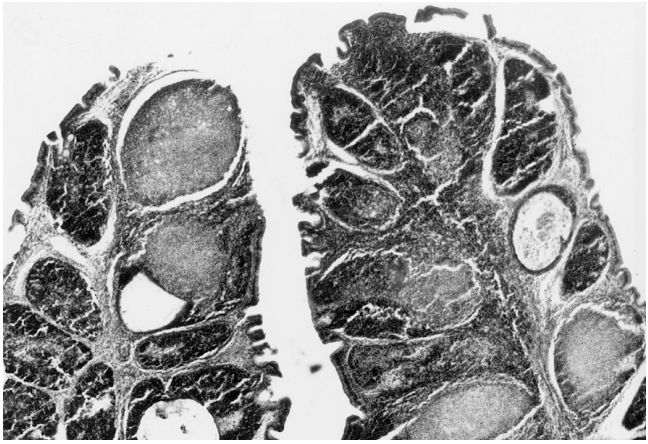

Figure 2. Photomicrograph of bursa Fabricii in chicken with spontaneous mycotoxic nephropathy. Degenerative changes, necroses and cyst formations in the lymph follicles. H/E. 100×.

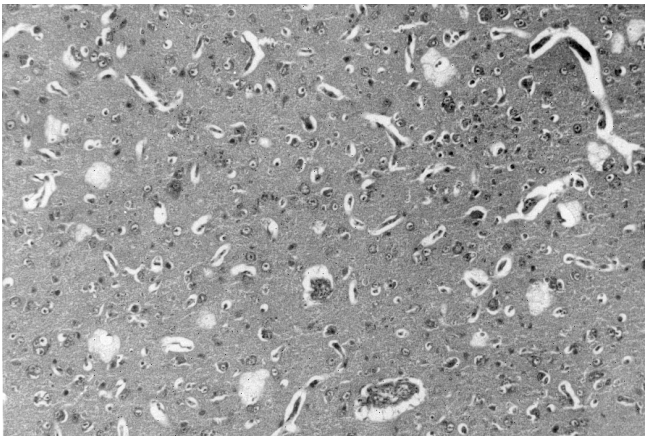

Figure 3. Photomicrograph of brain in chicken with spontaneous mycotoxic nephropathy. A pericellular or pericapillary oedema and lytic changes in ganglionic and glia cells. H/E. 200×.

3.6. Ultrastructural findings

The ultrastructural investigation of kidneys showed that the major lesions were observed in the epithelial cells of the proximal tubules. The brush border was reduced in height and density. The quantity of cell organelles in the basal part of the damaged epithelial cells was decreased. Many cell organelles showed damage of the membranes. Mitochondria were often swollen with electron dense formations and lipid drops; their cristae were damaged or reduced in number (Fig. 4). The nuclei were often enlarged and vesicular or diminished with aggregation of chromatin. A large number of collagen fibrils in the interstitium and thickened basement tubular membranes were rarely observed. The main changes in hepatocytes were also observed in the mitochondria. They were swollen, with damaged and reduced cristae, lipid drops, and in some cases showed a loss of membrane integrity.

Ultrastructural examination of the kidney and liver in control chickens did not reveal significant pathological changes similar to the ones described.

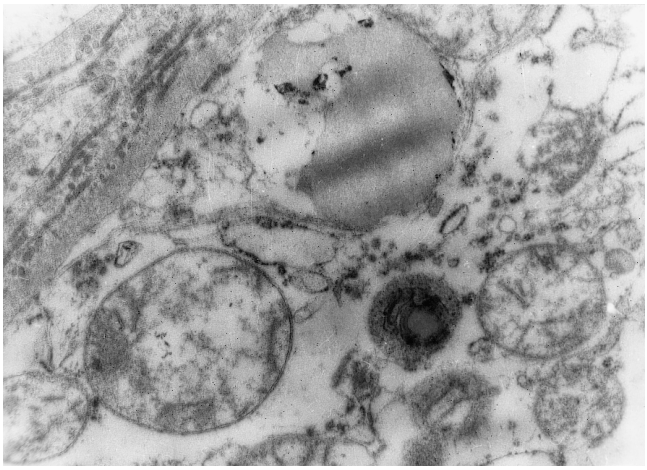

Figure 4. Electron micrograph of the epithelium of the proximal tubules of a kidney in chicken with spontaneous mycotoxic nephropathy. Swollen mitochondria with damaged or reduced cristae and lost membrane integrity. Lipid mass in the cytoplasm. 32 000×.

Table II. Mean (± SEM, $n = 4$) concentration of ochratoxin A (OTA) in kidney and liver samples of chickens from farms with and without nephropathy.

% of nephropathy in studied farms	Number of OTA positive samples	Mean OTA in positive samples (ng/g)	Mean OTA in all studied samples (ng/g)
kidney			
0	0	0	0
40–50	3	9.03 ± 0.43	6.78 ± 2.28
40–50	3	9.93 ± 0.88	7.45 ± 2.56
liver			
0	0	0	0
40–50	3	10.66 ± 1.13	8.00 ± 2.78
40–50	3	7.23 ± 1.69	5.42 ± 2.17

3.7. Toxicological findings

A significant ($p < 0.05$) difference was found between the quantities of OTA in the kidneys and livers of chickens with high frequency of nephropathy and chickens without nephropathy (Tab. II), but the difference was not significant between serum levels of OTA in chickens with high frequency of nephropathy and those without nephropathy (Tab. III).

A close relationship was also seen between the frequency of nephropathy and the contamination levels of OTA in the muscles (Tab. IV).

All of the feed samples originating from 5 chicken farms with nephropathy, were positive for OTA; the values ranged from 90.8 to 310 ng/g (mean 196.2 ng/g ± 45.9). No OTA was found in the feed samples from farms without nephropathy.

Table III. Mean (± SEM, $n = 5$) concentration of ochratoxin A in serum samples of chickens from farms with and without nephropathy.

% of nephropathy in studied farms	Number of OTA positive samples	Mean OTA in positive samples (ng/g)	Mean OTA in all studied samples (ng/g)
0	0	0	0
about 40–50	1	0.28	0.056 ± 0.05
about 40–50	1	1.42	0.284 ± 0.28

Table IV. Mean (± SEM, $n = 15$) concentration of ochratoxin A in muscle samples of chickens from farms with different frequencies of nephropathy.

% of nephropathy in studied farms	Number of OTA positive samples	Mean OTA in positive samples (ng/g)	Mean OTA in all studied samples (ng/g)
1–2	1	4.7	0.31 ± 0.31
40–50	6	4.16 ± 0.33	1.67* ± 0.56
above 80	10	4.68 ± 0.46	3.12** ± 0.66

* Significant difference compared to chickens with 1–2% nephropathy ($p < 0.05$).
** Significant difference compared to chickens with 1–2% nephropathy ($p < 0.001$).

4. DISCUSSION

The significant decrease in the serum total protein suggests that OTA could impair protein synthesis in broiler chickens as has been previously found [20, 28, 29]. The significant increase of the serum levels of uric acid shows that the kidney function was severely impaired, which may additionally contribute to the decrease of the total serum protein. The low serum level of cholesterol is most likely due to the decreased energy production necessary for its biosynthesis in the liver [18].

Growth depression in chickens according to some authors [20] is due to disorders of protein synthesis, provoked by OTA.

Histopathological changes correspond well to clinical signs (including nervous symptoms) and clinical biochemistry. Degenerative changes of the epithelial cells of kidneys and the liver are probably due to the route of elimination of OTA via the kidneys and partly via the liver, due to enterohepatic recirculation and hepatobiliar way of excretion of OTA [9], provoking a direct toxic effect of OTA on these organs [29]. The major lesions of the kidneys were observed in the mitochondria of the epithelium of proximal tubules as previously reported [4, 29], whereas the proliferative changes in the interstitium were rarely manifested, probably as a consequence of degenerative lesions.

Degenerative changes and decreased lymphoid tissue in the bursa of Fabricius, thymus and spleen are in good agreement with some recent observations in experimental MNC provoked by OTA, reporting the suppression in cell-mediated [7] and humoral immune responses [6, 13]. Commonly, an immunosuppressive effect of OTA has been observed at feed levels of 2 ppm OTA [23], but our recent observation revealed that significantly lower feed levels of OTA (0.13–0.79 ppm) could also have an

immunosuppressive effect, when received in combination with penicillic acid [29]. In natural conditions OTA is only part of a complex array of mould metabolites, such as penicillic acid, which may be synergistic with it in immunosuppression. The extent to which OTA may interact with other components of commercial chicken rations compounded from agricultural produce may also influence the significance of the relatively lower doses of OTA that commercial chickens may encounter in some feed. Moreover, OTA induced nephropathies in pigs or chickens have been previously related to an increased sensibility to bacterial infections [8, 30], which could additionally complicate the clinicomorphological picture of these nephropathies.

Oedema of the brain and cerebellum [12, 29] as well as various subcutaneous [29] or liver and kidney [3, 24] haemorrhages might be due to OTA-induced vascular damages [29], but these have not been observed in MNC described in Scandinavia [5].

A close relationship has been observed between the frequency of MNC and the rate of OTA in muscles of chickens, but the levels of OTA in corresponding feed samples (0.09–0.31 ppm) were significantly lower than the levels (2–4 ppm OTA) [3, 16, 20] required to reproduce such nephropathy. Moreover, some of the changes of Bulgarian MNC described such as necroses or cyst formation in lymphoid organs have not been reported in spontaneous MNC described in other countries, because such changes could be expected only at significantly higher feed levels of OTA. In fact, the same changes could be due to some synergistic effects between OTA and other mycotoxins, and the increase of OTA-toxicity [29]. Degenerative and weight changes in kidneys and lymphoid organs as well as changes in the serum levels of uric acid were recently seen in chickens at only 0.2 or 0.3 ppm OTA in combination with penicillic acid using a mouldy diet [19, 28, 29], whereas such changes had been seen in chickens exposed to significantly higher levels of pure OTA (about 4 ppm) in feed [3, 16, 20]. It is known that penicillic acid inhibits in vitro and in vivo pancreatic enzyme carboxypeptidase A [22], the enzyme responsible for detoxification of OTA in the small intestine. Recently, it has been found that the toxicity of various strains of the same *Aspergillus ochraceus* group is very different, independently of the capacity of OTA production [28, 29, 31].

A previous study of the fungal origin of OTA does not support the assumptions that the conventional fungi (*Penicillium verrucosum* and/or *Aspergillus ochraceus*) are the sources [17, 26]. Mycological investigations of animal feeds in Bulgaria revealed the presence of a *P. aurantiogriseum* complex [26], which is a potent producer of penicillic acid. Therefore, it is already of great importance to carry out more mycological investigations of feeds in order to isolate the ochratoxinogenic fungi in Bulgarian feeds and to produce some experiments using mouldy feed containing OTA and other (probably unknown) mycotoxins produced by the same ochratoxinogenic fungi as occurs in the field, because such experimentally moulded feed shows significantly increased toxicity of OTA in chickens [28, 29] than pure OTA. The production of multiple toxins by a single fungus or by a mixture of fungi presents a problem that has not been sufficiently investigated. Often such mixtures of toxins have additional or synergistic effects in farm animals, greater than would be expected from the action of any single toxin. This could explain why the low levels of OTA in Bulgarian feeds for pigs [26], chickens or humans [25] have such high toxic effect on kidneys, when received by spontaneous mouldy feeds.

Because of the assumption that all chickens investigated from the same batch had been fed the same feed, the presence of some damages (macroscopic or histological) in kidneys and OTA content in all investigated

tissue samples from this batch would be expected. Thus, the gregarious biochemical or toxicological investigations in chickens present a good base for diagnosis of this nephropathy. Also, it is difficult to assess OTA contamination of various feeds because of the pronounced variation in OTA contamination of feedstuffs even in the same crop from the same area. The main reason for the pronounced variation in results of monitoring feedstuffs for OTA contamination is the circumstance that the fungi invade only a minor fraction of feed particles with appropriate condition for growth of fungi and OTA formation [10]. Thus, OTA contamination of feedstuffs in two closely situated places is markedly different. This explains why not all of the chickens from the same farm fed with the same feed had kidney damages or OTA-content.

Because of the assumption that mycotoxins and especially OTA are involved in the human renal disease Balkan endemic nephropathy [14, 25] the exposure of humans to this very hazardous and relatively heat stable toxin from chicken meat needs to be prevented. A good preventive measure is the toxicological analysis of a few blood samples from chicken farms suspected of MNC a week before slaughter and a change in the feed source for a week if it is necessary. Also, the period of feed deprivation of chickens before slaughter could be prolonged. Because of the short half-life of OTA in chickens (4 hours) [21] its concentration in blood and various tissues quickly decreases after changing the feed source or after prolonging the period of feed deprivation of chickens before slaughtering.

Keeping in mind the low contamination levels of OTA in serum, tissue and feed of chickens with nephropathy, we can conclude that the chicken nephropathy, similarly to porcine nephropathy in Bulgaria [26, 27], may have a multitoxin or multifactor aetiology, because it cannot be explained by the concentration/action of OTA alone. Detailed investigations have to be made to identify the cause of nephropathy in chickens.

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