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Morphological and Functional Evaluation of Chicken Blood Leukocytes in Chronic Ochratoxicosis

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Abstract: The effects of ochratoxin A (OA) administration on the function and morphology of chicken blood leukocytes was evaluated. Two-weeks-old, broilers chickens (Cobb-500) were randomly divided into three groups, six birds each. The birds form the group I received a fodder without ochratoxin. The chickens from the group II were fed with fodder containing 6ppm of ochratoxin A for 10 days, while administration of ochratoxin to the birds from group III was prolonged up to 20 days. After 10 days of administration the OA containing fodder, there was recorded the increase of hematocrit (PCV) and characteristic for stress, decrease of lymphocyte percentage with simultaneous increase of heterophils percentage. The prolonged administration of the OA containing fodder to the subsequent 10 days, lead to the withdraw of the mentioned leukogram changes, decrease of PCV value and decrease of hemoglobin (Hb) level. Simultaneously, there was demonstrated that the heterophils of the chickens receiving OA, characterized with increased phagocytosis ability of the yeast cells (17% of phagocytizing cells in the group I, while in group II and III 26 and 37% respectively). However, the lymphocytes of these birds, after their incubation with oxalates mixture, characterized with decreased ability of radial segmentation of their nuclei (11.4% in the group I, 8% in the group III). The obtained results show not only OA immunosuppressive activity mechanisms, but also indicate the dissimilar or even very different sensitiveness of the particular leucocytes.

Key words: Chickens, ochratoxin A, phagocytosis, radial segmentation of lymphocyte nuclei

Introduction

Storing fodder and feeding products in unfavorable environmental conditions leads to fungi growth and occurrence of considerable quantities of their metabolites - mycotoxins. Wide spectrum of micotoxins biological effects (toxic, mutagenic, teratogenic, and eventually estrogenic), as well as the fact that they outstanding resistance to temperature make their presence in fodder a serious problem, especially in poultry and herd breeding (Marquardt and Frohlich, 1992). Micotoxins like aflatoxins, ochratoxins, fumonisins or zearalenone are produced mainly by fungi of genus *Aspergillus*, *Penicillium* and *Fusarium*. Ochratoxin A (OA) is known because of its special nephrotoxic and hepatotoxic activity (Dwivedi and Burns, 1984; Castegnaro *et al*., 1991; Baudrimont *et al*., 1994; Santin *et al*., 2002; Petrik *et al*., 2003) connected, among the others, with protein synthesis inhibition, generation of free radicals leading to enhanced lipid peroxidation and DNA damage, inhibition of both mitochondrial respiration and ATP synthesis (Haubeck *et al*., 1981; Höhler, 1998; Wang and Groopman, 1999). Ochratoxin A also induces the changes in lymphatic organs in birds (mainly in bursa of Fabricius) and disturbs leukocyte function, which results in immunosuppression (Huff *et al*., 1974; Singh *et al*., 1990). However, immunosuppression mechanisms accompanying ochratoxicosis, as well as changes in immunological system have not been well-known so far. Taking in to account the above facts this investigation focused on lymphocyte and heterophils reactivity regarding their different contribution to the process shaping the immunity of an organism. To this end, in chickens exposed to ochratoxin A in fodder there was applied a phagocytic test to evaluate the ability of heterophils, to ingest yeast cells. At the same time, the lymphocyte cytoskeleton status was evaluated indirectly by inducing the radial segmentation of lymphocyte nucleus (RS), aiming at determination whether OA affects the function of both groups of leukocytes in the same way.

Materials and Methods

18 chicken broilers COB-500 hybrids, two-weeks - old, kept in farm condition were the subject to the experiments. Six birds constituted a control group (I) were fed with mycotoxins free standard fodder. The birds of group (II) were fed with fodder containing 6 ppm of ochratoxin A for ten subsequent days. In group (III) administration of fodder with the same quantity of OA lasted for twenty days. Within the whole period of the experiment the quantity of fodder and water intake was the subjected to a strict control. Ochratoxin A (OA) was produced in the course of fermentation of wheat seeds with *Aspergillus ochraceus*, strain KA-10, within the period of 192 hours at 28°C. After fermentation, the wheat was dried. The culture obtained containing 102,4 mg/kg of OA, was mixed with a standard, mycotoxin - free fodder until it ranged a final

concentration of 6 ppm of OA.

The blood was collected after the tenth and the twentieth day of the experiment. There was determined hemoglobin level (Hb) and the hematocrit value (PCV). The smears were the basis to establish leukogram, counting up to 200 the leukocytes found. The ability of granulocytes to phagocytosis was defined according to Kreukniet *et al.* (1995) involving incubating the mixture of blood and yeast suspension in water bath at 41°C for 30 min. The smears prepared after incubation were stained using May-Grünwald-Giemsa method. The percentage of phagocytic cells was evaluated, using an optic microscope, by counting found heterophils up to 200. At the same time the remaining part of collected blood samples was used to test radial segmentation of lymphocyte nuclei (RS). The test followed the method by Söderström for spontaneous and induced radial segmentation. In its modified version (Graczyk and Pliszczak-Król, 1996) 0,2 ml of oxalate mixture (solution of 0,57% potassium oxalate and 0,85% ammonium oxalate mixed 1:1) was added to 0,8 ml of heparinized blood and incubated for 3 hours at room temperature (22°C). Afterwards, the samples were centrifuged and leukocyte layer situated between erythrocytes and plasma was collected. 3 smears were prepared out of each chicken. Then the smears were fixed in methanol and stained using May-Grünwald-Giemsa method. The following step involved differentiation between RS positive (RS +) and RS negative (RS-) lymphocytes (the lymphocytes found were counted up to 200), according to criteria described earlier (Graczyk and Pliszczak- Król, 1996; Pliszczak-Król, 2001). The Student t-test with paired samples was used to compare and analyze the data. A p value of <0,05 between groups is considered significant.

Results and Discussion

The research carried out proved that after ten days of feeding the chickens with fodder containing OA the value of PCV and Hb concentration slightly increased (Table 1). The control of birds daily water intake showed its considerable reduction. As the changes in blood seemed to be significant and water intake relation, mentioned above, remained comparatively low, it is not possible to unanimously state whether the increase in the discussed values was the result of dehydration following kidney damage or the effect of a diminished water intake. Those data seem to be quite interesting, especially since prolonged feeding with fodder containing OA resulted in decreased PCV value and Hb content, accompanied by low water intake, but not in their further increase. It is probably connected not only with kidney damage but also with disorder in hematopoiesis (Huff *et al.*, 1979). Those records correspond with earlier findings regarding the occurrence of anemia in the course of ochratoxicosis (Huff *et al.*, 1979; Ayed *et al.*, 1991).

Considerable decrease in both Hb content (from 7,3 g/dl in group I to 6,2 g/dl in group III) and PCV value (from 31,1% in group I to 26,8% in group III) speak for the above interpretation (Table 1). The data obtained due to the analysis of leukocyte pattern of experimental chickens seem to be interesting as well. After ten days of feeding chickens with fodder containing ochratoxin there was recorded the increase in percentage of heterophils, accompanied by the decreased percentage of lymphocytes (Table 1). Such a behavior of leukocytes in blood is similar to the changes characteristic for adaptation processes, namely for stress (Gross and Siegel, 1983; Graczyk, 1999). The mentioned pattern of alterations resembled the one observed by Chang *et al.* (1979) and Singh *et al.* (1990) who pointed out simultaneous decrease in total leukocyte number and unchanged or reduced heterophils number. However, further feeding with this mycotoxin leads to complete regression of the changes observed. Therefore, it can be supposed that ten-days`-period of ochratoxin application causes, apart from specific changes in immunological system, unspecific changes as well, which are expressed by the described alterations in blood pattern. Sustained ochratoxin administration is followed by adaptation to an active stressor, while the changes in blood undergo regression. This means that ochratoxin present in fodder, apart from its toxic effect, also affects the organism as a stressor of an environmental origin. Such an activity of mycotoxins was also reported by Slowik *et al.* (1993) in their investigation on aflatoxin B_1. Thus, taking into account immunosuppressive effects of mycotoxins from fodder, the following question can be formulated: whether and to what degree immunosuppression in the course of mycotoxicosis is exclusively a result of toxic effects of mycotoxins or if it is also a result of an additional stress. To answer that question a detailed evaluation of specific and unspecific immunological mechanisms is required.

The efficiency of leukocytes is not only evidenced by their presence in blood, but, first of all, by their quality and ability to actively participate in defensive processes in the organisms. The attempt of evaluation of heterophils efficiency in chicken was undertaken using the test of yeast cells phagocytosis (Table 2). Contrary to common expectations, it turned out that ten and twenty days of OA administration the percentage of heterophils containing yeast cells did increase from 17,6% in the group I to 26,7% and to 37% in both experimental groups. The interpretation of that phenomenon is extremely difficult since the literature data usually present an inhibitory activity of mycotoxins, or the one which does not change their phagocytic activity (Chang and Hamilton, 1980; Campbell *et al.*, 1983). However, considerable differences between the recorded values allow to state that OA administered to the chickens can demonstrate

Table 1: Hematological indices in chicken blood in chronic ochratoxicosis

Group	HbG/dl	PCV %	Heterophils %	Lymphocytes %
I	7.3 ± 1.41	31.1 ± 2.76	33.0% ± 0.09	60.8% ± 0.08
II	7.6 ± 2.98	34.6 ± 1.33***	40.3% ± 0.04***	52.0% ± 0.03***
III	6.2 ± 1.34**	26.8 ± 4.09***	29.0% ± 0.16	63.5% ± 0.18

Table 2: Phagocytizing heterophils and RS+ lymphocytes in chicken in chronic ochratoxicosis

Group	Phagocytizing heterophils %	RS+lymphocytes %
I	17.6% ± 0.07	11.4% ± 0.04
II	26.7% ± 0.07***	10.3% ± 0.04
III	37.4% ± 0.09***	8.0% ± 0.04**

Legend: I - Control group, II - chicken fed with fodder containing ochratoxin A for 10 days
III - chicken fed with fodder containing ochratoxin A for 20 days, *- statistically significance difference $p<0.05$
- statistically significance difference $p<0.01$, *- statistically significance difference $p<0.001$

stimulation of phagocytosis.

On the other hand, time efficiency of phagocyted material degradation becomes, probably, decreased. Such a view can be supported by the decrease in NBT - positive heterophils quantity under the influence of ochratoxin, reported by the numerous authors, suggesting a disordered intracellular process of phagocytized material destruction (Singh *et al.*, 1990). The above interpretation agrees with the data regarding an inhibitory influence of OA on oxygen reactions in cells. (Fink-Gremmels *et al.*, 1995; Höhler, 1998; Petrik *et al.*, 2003), as well as with the fact of a decrease reactive oxygen compounds production resulting from aflatoxin B_1 activity (Moon *et al.*, 1999).

Evaluation of heterophils phagocytic activity was accompanied by investigation on lymphocytes ability to form radial segmentation of their nuclei (RS). The reports by numerous authors indicate that forming lymphocyte RS nuclei expresses the dominance of microtubule depolimerization of a cell cytoskeleton. The latter process results from the effect of different agents and leads to the occurrence of deep clefts in cell nuclei, which, in turn, divide nuclei into several segments and make them look like clover leaf or figure eight (Graczyk, Pliszczak-Król, 1996; Pliszczak-Król, 2001). This phenomenon appears spontaneously or it can be induced by incubating blood with the addition of oxalates mixture. Yet a precise mechanism of RS emergence has remained unknown. Some authors presume that induced RS affects only those cells which while changing their activity, accumulate considerable amounts of calcium and simultaneously become more sensitive to its loss (Ito, 1974). The research proved that, regardless individual differences, ochratoxin A inhibits lymphocytes ability to form oxalates-induced RS. Although this fact seems to be difficult to precisely explain, it agrees with the suggestion by Gentles *et al.* (1999) that ochratoxins disrupt calcium homeostasis in cells. On the basis of the research carried out it can be assumed that lymphocytes remain especially sensitive to this kind of OA activity. The mentioned changes of percentage of RS cells can also be related to the decrease in lymphocyte number as the effect of OA induced apoptosis (Seegers *et al.*, 1994a; 1994b, Petrik *et al.*, 2003). RS inhibition rate seems to be dependent on the time of ochratoxin activity. Although RS was noticeable in 11,4% of control group I, after 10 days its level decreased to 10,3% and then to 8% after 20 days (Table 2). This ochratoxin shows a diversified effect on different populations of blood leukocytes, since it induces an increase in heterophils phagocytic activity, and the same time, a decrease in lymphocytes ability to form radial segmentation of their nuclei, which exemplifies structural and functional differences of those cells. The data obtained, pointing out diversified immunosuppressive effects of ochratoxin A, encourage further investigation on ochratoxin A immunosuppressive effects.

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