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Effects of aflatoxin B_1 on the erythrocyte count, the content of hemoglobin, and the immune adherence function of erythrocytes in chickens[1)]

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Summary

One hundred and twenty one-day-old male Avian broilers were randomly divided into four equal groups and fed for 21 days as follows: a control diet and three aflatoxin B_1 supplemented diets containing 0.15, 0.3, and 0.6 mg/kg aflatoxin B_1. The RBC count, the content of hemoglobin, and the immune adherence function of erythrocytes were determined. The results in the three aflatoxin B_1 groups indicated that the RBC count was increased, the rate of C_3b receptor rosette and the content of hemoglobin were decreased, whereas the rate of immune complex rosette showed no obvious change compared with the control group. These results show that aflatoxin B_1 induced primary damage to the erythrocytic adherence function.

Keywords: aflatoxin B_1, C_3b receptor rosette rate, immune complex rosette rate

Aflatoxins (AFs) are secondary toxic metabolites produced by certain fungi, mainly *Aspergillus flavus* and *Aspergillus parasiticus*. Immunosuppression caused in chickens by small doses of aflatoxins, especially aflatoxin B_1 (AFB_1), has been reported in many sources (6, 15, 16, 19, 29, 32, 37). It is characterized by reduced antibody titres, leucopenia, a repressed development of the thymus and the bursa of Fabricius, suppression of phagocytosis, and the subsequent diminution of antigen presentation by macrophages. At the same time, some hematological parameters, such as the count of RBC, Hb (hemoglobin), and others, also changed (9, 12, 32), indicating that erythrocytes are to some extent affected by aflatoxin. Although macrophages may be damaged by aflatoxin, considering that erythrocytic immune adherence plays an important role in the efficient transport of antigen-antibody-complement complexes for clearance by the fixed macrophage system (7, 35), we suspect that the toxic damage to erythrocytic immune adherence caused by aflatoxin may partly contribute to the whole immunosuppression.

However, relevant information on the impact of AFB_1 on hematological changes is scarce, and there have been no studies on its impact on the immune function of erythrocytes in chickens. Therefore, we conducted an experiment to detect changes in the red blood cell count, hemoglobin content, and erythrocyte immune function in Avian broilers exposed to AFB_1, hoping to supplement the aforementioned relevant information.

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Material and methods

Animals and diets. Male Avian broilers weighing 45 ± 5 g were purchased from a commercial rearing farm (Wenjiang poultry farm, Sichuan province). A total of 120 one-day-old broilers were randomly divided into control and three AFB_1 groups, and kept in alloy cages under standard conditions. Aflatoxin B1, obtained from Fermentek Ltd (Jerusalem, Israel, 1162-65-8), 1.5, 3, 6 mg AFB_1 farinose solid was completely dissolved in 30 mL dimethyl sulfoxide. Then the 30 mL mixture was mixed into a 10 kg corn-soybean basal diet to formulate the AFB_1 diets of the experimental groups, containing AFB_1 0.15 mg/kg (group I), 0.3 mg/kg (group II), and 0.6 mg/kg (group III). The equivalent dimethyl sulfoxide was mixed into the corn-soybean basal diet to formulate the control diet. Then the dimethyl sulfoxide in diets was evaporated at 98°F (37°C) for 3 days. Broilers were provided with drinking water and diets *ad libitum* for 21 days. The use of broilers and all experimental procedures involving animals were approved by the Sichuan Agricultural University Animal Care and Use Committee.

Nutritional requirements were adequate according to the National Research Council (25) and Chinese Feeding Standard of Chicken (NY/T33-2004).

Total RBC count and Hb content. At 7, 14, and 21 days of the experiment, blood samples were collected by vein puncture (jugular vein) from 5 broilers in each group and anticoagulated with 40 g/L of Ethylene Diamine Tetraacetic Acid (EDTA). Blood samples of 20 µL were diluted with 2 mL normal saline. Appropriate suspension was then added along the edge of a clean blood counting plate covered with a cover slip. After 2 minutes, the red blood cell numbers were recorded under an Olympus microscope (Japan) to check if the density was within the proper range.

0.1 mol/L hydrochloric acid was dripped into the hemoglobin determination tube to reach a scale of "2" or "10%". 20 µL blood was gently added to the tube and mixed with hydrochloric acid. After 10 minutes, distilled water was added until the color was the same as that of the standard plate. The marker of the liquid meniscus denoted the Hb content in g/100 mL blood.

Erythrocyte immune function. 500 µL blood was added into a centrifuge tube. After washing three times, red blood cells were suspended to a concentration of 1.25×10^7/mL with saline. Complement-coated yeast and uncoated pure yeast, purchased from the Immunology Department of the Shanghai Changhai Hospital, were used for detecting C_3b receptor rosette (C_3bRR) and immune complex rosette (ICR), respectively.

The complement-coated yeast was washed and suspended to a concentration of 1×10^8/mL with saline. 50 µL red blood cell suspension and 50 µL complement-coated yeast suspension were added into a 5 mL tube and mixed gently. After the mixed solution was incubated at 37° for 30 minutes, 25 µL of 0.25% glutaraldehyde was added to fix the cells. Subsequently, 25 µL mixtures were spread on a microscope slide and stained with Giemsa staining solution. Two hundred red blood cells were counted under a microscope. A red blood cell binding with two or more yeast cells was counted as one rosette. The ratio of red blood cell C_3b receptor rosette (C_3bRR) was then calculated.

The ratio of immune complex rosette (ICR) of red blood cells was determined by the same procedure, described above, by replacing the complement-coated yeast suspension with an uncoated one.

Statistical analysis. Statistical analysis was performed with SPSS (Statistical Product and Service Solutions) 16.0 for Windows. All parameters determined in this study are presented as mean ± standard deviation ($\bar{x} \pm$ SD). Statistical analysis was performed by the one-way analysis of variance (ANOVA) test. A probability value of $p < 0.05$ was considered as significant difference.

Results and discussion

RBC count and Hb content. As shown in Figure 1, with the increasing content of AFB_1 in the diets, the RBC count was increased, and the hemoglobin content was decreased. At the age of 7 days, the RBC counts in AFB_1 groups I, II and III were higher or significantly higher than the RBC count in the control group ($p < 0.01$ or $p < 0.05$), whereas the Hb contents in AFB_1 groups II and III were lower than in the control group ($p < 0.05$). At the age of 14 days, there were no significant differences in the RBC count among the four groups, but the Hb content in AFB_1 group III was lower than it was in the control group ($p < 0.05$). At the age of 21 days, the RBC count in AFB_1 group III was significantly higher or higher than those in the control group, AFB_1 group I and AFB_1 group II ($p < 0.01$ or $p < 0.05$), whereas the Hb contents in AFB_1 groups II and III were lower than the Hb content in the control group ($p < 0.05$).

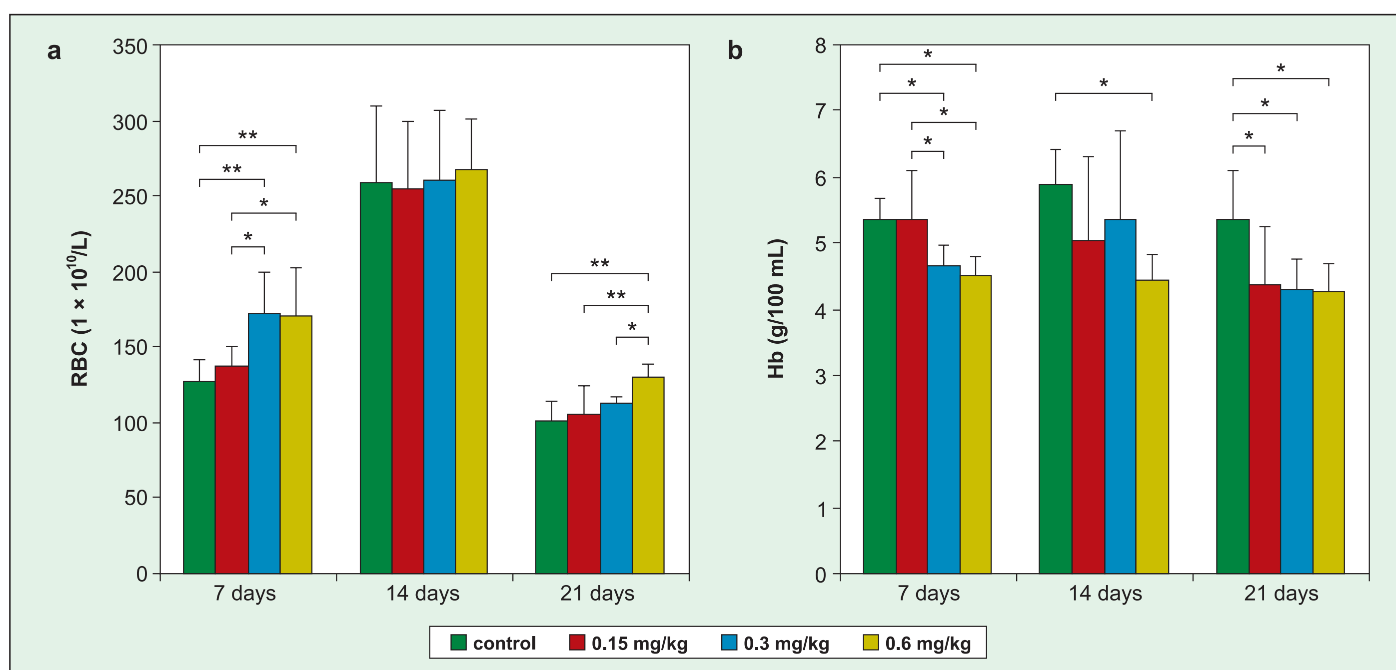

Fig. 1. The RBC count and Hb content in chickens in different experimental groups (n = 5)
Explanation: Comparison among groups: * $p < 0.05$; ** $p < 0.01$

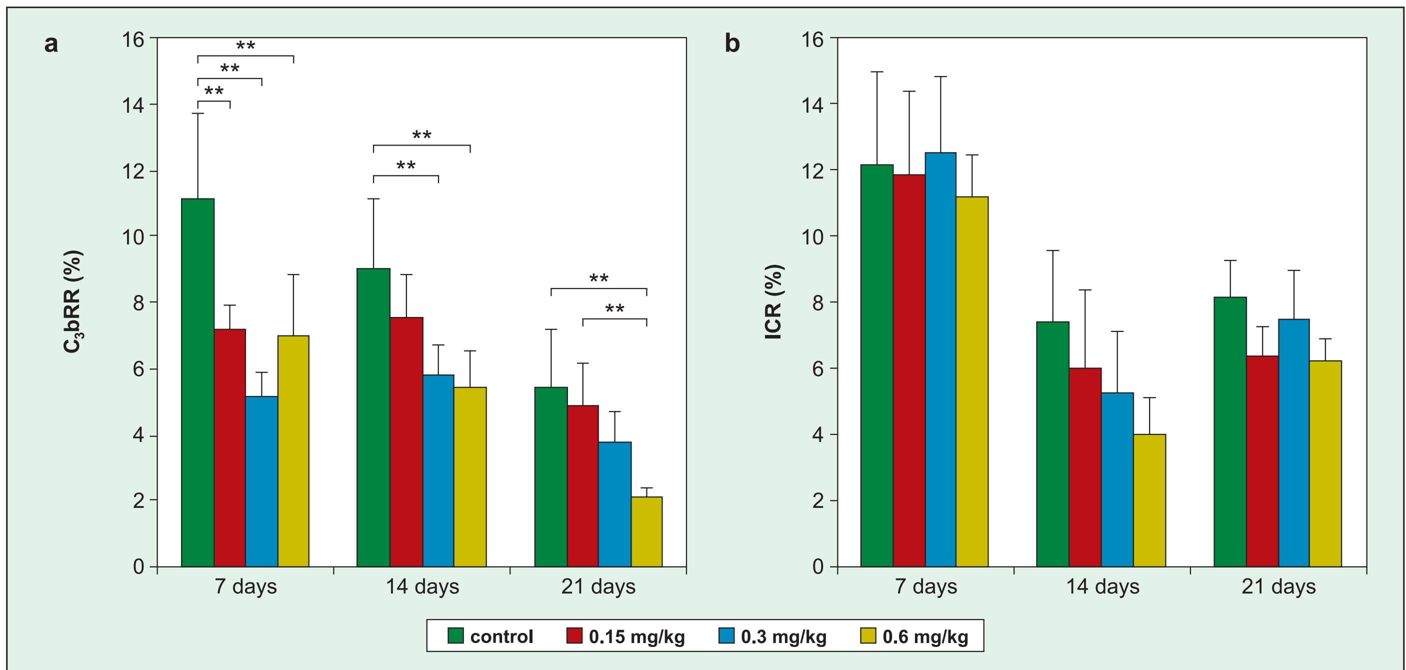

Fig. 2. The rates of erythrocyte C_3bR rosette and IC rosette in chickens in different experimental groups (n = 5)
Explanation: Comparison among groups: * p < 0.05; ** p < 0.01

Erythrocyte C_3b receptor rosette rate (C_3bRR) and immune complex rosette rate (ICR). As shown in Fig. 2, the C_3bRR decreased with the increasing content of AFB_1 in the diets. The C_3bRRs in AFB_1 groups I, II and III were significantly lower than the C_3bRR in the control group ($p < 0.01$) at 7 days of age. The C_3bRRs in AFB_1 groups II and III were significantly lower than the C_3bRR in the control group ($p < 0.01$) at 14 days of age, and the C_3bRR in AFB_1 group III was significantly lower than the C_3bRR in the control group ($p < 0.01$) at the age of 21 days. The ICR of erythrocytes showed no obvious change, and no significant differences were noted among the four groups at 7, 14, and 21 days of age ($p > 0.05$).

The reasons for the levels of AFB_1 in feed. In this study, aflatoxin was administrated at 3 dose levels: 0.15 mg/kg, 0.3 mg/kg and 0.6 mg/kg. According to reports by Chen X et al. (6) and Ghosh RC et al. (15), the threshold dose of AFB_1 eliciting immunotoxicity is approximately 0.3-1.0 mg/kg. Therefore, the 3 dose levels were selected to induce erythrocytic immnosuppression and to determine whether a dose smaller than 0.3 mg/kg can also lead to the immune damage of erythrocytes or not. Besides, with reference to some literatures (3, 5, 20, 31, 38) and the climate of Sichuan province – warm and moist, these dose levels probably exist in the normal chicken's diets of poultry industry, especially in corn/peanut basal diets (20).

Effects of AFB_1 on the RBC count and the content of Hb. The experimental results show that the RBC count is increased and the content of Hb is reduced with a rising dietary concentration of AFB_1. Changes in the RBC count and Hb content have been reported in some animals exposed to AFB_1. Edrington et al. and Fernandez et al. (10, 12) found that the RBC count was significantly increased, whereas the content of Hb was significantly decreased when lambs ingested 2.5 mg/kg AFB_1 for 20 days. All our results tend to be in accordance with the above research. In the present study, the RBC count after 14 days was significant higher than after 7 days and 21 days, which may have been caused by controllable or uncontrollable factors related to blood processing, chicken, chicken diet etc. and so on (25, 36). Simultaneous examinations of the RBC count and Hb content can roughly reflect the type and degree of anemia, and the Hb content is positively correlated with the RBC count in general (8). But in our study, the RBC count was negatively correlated with the Hb content. This situation often occurs in the early stage of iron-deficiency anemia (28), and a decrease in serum iron ion level has been observed in lambs fed with AFB_1 (10). However, the changes in these clinical blood values reported by Edrington et al. and Fernandez et al. (10, 12) are not conclusive with reference to similar studies by Fernández et al. (13) on lambs, by Dönmez et al. (9) on Merino rams, and our own. Moreover, a study by Lanza et al. (23) suggests that differences among chicken breeds could also affect changes in blood parameters during a dietary aflatoxin experiment. Besides serum iron difficiency, the inhibition of protein synthesis caused by aflatoxin may contribute to anemia. Studies by Whipple (39) indicate the hemoglobin synthesis and erythrocyte production are maintained preferentially at the expense of other body proteins. And the hypoproteinemia associated with aflatoxin was reported by Lanza et al. (23) and Agha et al. (1). Considering the anemia type observed in our study, we suspect that the synthesis of iron meta-

bolic protein, especially transferrin, may be impeded by aflatoxin, which can deliver iron to red blood cell precursors to product red blood cells (24).

In addition, by stimulating phospholipid A_2 to initiate lipid peroxidation in cells, AFB_1 metabolites may disturb the integrity of the erythrocytic membrane, which consequently decreases erythrocyte deformability and increases erythrocyte viscosity (4, 33). Collectively, Taking together, because of iron-deficiency anemia and viscous erythrocytes, the transport capacity of erythrocytes was impaired in chickens exposed to AFB_1, which could have induced an increase in erythrocytes resulting from a compensatory mechanism.

Effects of AFB_1 on the immune function of erythrocytes. Recent evidence suggests that erythrocytes are natural immunocytes, and the C_3b receptor (CR_1) on erythrocytes plays an important role in the efficient transport of antigen-antibody-complement complexes for clearance by the fixed macrophage system (7, 35). Our results show that the rate of C_3b receptor rosette was lower in the AFB_1 groups than it was in the control group. The results suggest that the erythrocytic immune adherence function is suppressed by AFB_1. The rate of C_3b receptor rosette is closely associated with the quantity and activity of CR_1 on erythrocyte membranes (27). CR_1 quantity is genetically determined, and its activity is influenced by the distribution of receptors on the erythrocyte surface. A clustered state and an un-clustered state are two CR_1 distributive patterns, but only the former can combine with complements to form stable multivalent bindings. Clarified toxicological mechanisms of AFB_1 include oxidative stress and, consequently, an impaired membrane system (34). In view of the aforementioned deduction and a discussion by the Eraslan et al. (11), oxidative stress will be aggravated as a results of the reduction of iron metabolic protein, which will cause an increase in free iron, playing a particularly important role in the Fenton reaction – one of the phases in lipid peroxidation (2, 22). Moreover, Patil et al. (29) reported that both the growth performance and the immune responses of chickens under experimental aflatoxicosis were improved by supplementation with melatonin or L-tryptophan (a precursor of melatonin), which is a potent antioxidant and scavenger of various free radicals, especially hydroxyl and peroxyl radicals, and could enhance the antioxidative enzyme activities in many tissues (30). On the basis of these opinions, in the present study, we suspect that the structure and number of CR_1 on erythrocyte membranes may be affected by oxidative stress.

It is important to note, however, that the rate of C_3b receptor rosette showed no significant difference among the control group, AFB_1 group I, and AFB_1 group II at the age of 21 days. This result suggests that older animals have a higher tolerance to the effects of AFB_1, which may be related to different metabolic speeds of AFB_1 in animals of different ages. It has been reported that AFB_1 is not toxic per se and requires metabolic conversion by Cytochrome P450s in liver microsomes to exert its toxicity (14, 17), and liver microsomes in younger birds are more active toward AFB_1 than those in older birds (21).

The rate of ICR in the AFB_1 groups was lower compared with that in the control group, but no statistical significance was observed. The rate of ICR is related to the content of circulating immune complex (CIC) and the activity of CR_1 on erythrocyte membranes (40). The primary damage to the erythrocyte immune function is due to a decrease or structural change in CR_1, and the secondary damage is attributable to the binding site occupied by CIC (18). In the present study, the rate of ICR was unaltered or decreased while the rate of C_3bRR was decreased, which reveals that the activity of CR_1 was suppressed and it could not combine with CIC effectively. These results show that the primary damage to the erythrocytic adherence function was induced in chicken after AFB_1 ingestion.

The results and the above discussion demonstrate that 0.3 and 0.6 mg/kg dietary AFB_1 can induce anemia with an increased RBC and decreased hemoglobin, as well as cause primary damage to the erythrocytic adherence function with a decreased C_3bR rosette rate and an unchanged IC rosette rate.

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