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Effect of Fusarium Contaminated Diet on Innate and Adaptive Immune Response in Rabbit Model

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Abstract: Fusarium mycotoxins have been shown to be potent immunosuppressive agents. The main objective of this study was to investigate the effects of chronic consumption of corn and wheat based diet naturally contaminated with Fusarium mycotoxins on innate immune indices. Normal diet, after addition of fusarium culture, was stored in a humid room. After storage and visible mold growth, contaminated diet was cultured and analyzed by HPLC method for confirmation of Fusarium contamination and mycotoxins level measurement, respectively. Twenty healthy male New Zealand white rabbits were used in this study. Animals were randomly divided to four treatment groups (4 per group): Groups consisted of: 1) control (normal diet), 2) Control + Vaccine, 3) Fusarium Contaminated diet, 4) Fusarium Contaminated diet + Vaccine. Blood samples were obtained at five time points: days 7, 14, 21, 28, 35 and 42 of the study. Comparison of phagocytosis percentage of contaminated diet group with control, from day 21 to 42, showed significantly ($P \leq 0.05$) lower percentage of phagocytosis. Serum IgG concentrations in mold contained diet decreased significantly only on day 21. The present study suggests that fusarium contaminated diet has an immunosuppressive effect through reduction of phagocytosis and IgG concentration. In the current study, the detrimental effects of fusarium contaminated diet on immune-response suggest that an accurate monitoring system for mycotoxins contamination in feeds is desirable for assurance of poultry health and welfare.

Key words: Fusarium · Mycotoxins · Immune Response

INTRODUCTION

Mycotoxins are considered to be unavoidable contaminants in foods and feeds and constitute a major problem all over the world [1]. Interest in these naturally occurring chemical compounds is intense due to their detrimental, sometimes carcinogenic, effect on human health, animal production and reproductive traits. More than 300 mycotoxins have been shown to induce signs of toxicity in mammalian and avian species [2] and this number is increasing.

Fusarium fungi are commonly found in temperate climates and Fusarium mycotoxins are likely the most economically significant grain mycotoxins on a global basis [1]. Fusarium mycotoxins, however, have also been found to contaminate pastures and forages. Among the trichothecene mycotoxins, deoxynivalenol (DON, vomitoxin) is a major contributor to reduced feed intake in animals. Trichothecene mycotoxins have also been shown to be potent immunosuppressive agents [3]. In this regard, immunotoxicity is considered to be the most common consequence of major mycotoxicosis [3].

The clinical symptoms of mycotoxin ingestion have been well characterized in domestic animals, poultry and laboratory animals and range from mortality to mild performance reduction. Consumption of some mycotoxins, at levels that do not cause overt clinical signs, suppresses immune functions and may decrease resistance to infectious disease [4]. The immuno-suppressive potency of various mycotoxins differs substantially. Effects of DON, 3-acetyldeoxynivalenol, fusarenon-X, T-2 toxin, ZEA, alpha-zearalenol, beta-zearalenol and nivalenol on T and B cells in a proliferation assay and antibody-dependent cellular cytotoxicity NK cell activity on human peripheral blood mononuclear cells have been studied [5]. Among the mycotoxins tested, T-2 toxin, fusarenon X, nivalenol and DON show the highest immuno-suppressing effect *in vitro* and mycotoxins-induced immuno-suppression is related to depressed T or B lymphocyte activity. Furthermore, they also inhibit NK cell activity [5].

The main objective of this study was to investigate the effects of chronic consumption of corn and wheat based diet naturally contaminated with Fusarium mycotoxins on innate immune indices.

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MATERIALS AND METHODS

Diet Preparation: Normal corn and wheat based diet, after addition of fusarium culture, was stored in a humid room with 26±2°C temperature. After 14 days storage and visible mold growth, contaminated diet was cultured and analyzed by HPLC method [6] for confirmation of Fusarium contamination and mycotoxins levels measurement, respectively.

Animal Preparation and Study Design: Twenty healthy male New Zealand white rabbits, weighing 2.0-2.5 kg, were used. The rabbits were kept for two weeks to be adapted to environmental conditions. They were housed in individual cages in a room with controlled: temperature 20±22°C and a 12 hour light/dark cycle. The rabbits were fed standard diet and provided with fresh water *ad libitum*. Animals were randomly divided to four treatment groups (4 per group): Groups consisted of: 1) Control (normal diet), 2) Control + vaccine, 3) Fusarium contaminated diet, 4) Fusarium contaminated diet + vaccine. The used vaccine was myxomatosis (Mixohipra-FSA®, Laboratorios Hipra, S.A. one dose/rabbit). Blood samples were obtained from the central ear artery using 22 G catheter at five time points: days 7, 14, 21, 28, 35 and 42 of study. Blood samples were placed into heparinized and none heparinized tubes. Plasma was separated and stored frozen until assayed. For immunological analysis, the serum was separated by centrifugation at 3000 rpm for 10 min and stored at -20°C until analysis.

Cell Separation: Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Paque gradient centrifugation according to the instructions of the manufacturer Pharmacia, Freiburg, Germany. Briefly, 4 ml of Ficoll-Paque gradient was pipetted into two 15-ml centrifuge tubes. The heparinized blood was diluted 1:1 in PBS and carefully layered over the Ficoll-Paque gradient. The tubes were centrifuged for 30 min at 1500 g. The cell interface layer was carefully harvested and the cells were washed twice in PBS and resuspended in RPMI 1640 supplemented with penicillin (50 U/ml)-streptomycin (50 g/ml) [7].

Yeast Particles: Frozen stored bakers' yeast (Saccharomyces cerevisiae) was autoclaved in PBS at 120°C for 15 min. after autoclaving, the yeast particles were washed in PBS and stored at 4°C. On the day of experiment the yeast particles were washed in Hanks balanced salt solution (HBSS) and counted using a hemocytometer, mixed with RPMI 1640 containing 10% rat fresh serum and incubated for 30 min at 37°C. Yeast particles were opsonized with rat fresh serum components. The yeast particles in RPMI 1640 were centrifuged for 10 min at 1500 g. Then the sediments were washed twice in HBSS. Sediments were solved in serum free RPMI 1640 and diluted to 107/ml [8].

Phagocytosis Assay: A sterile glass cover slip placed in each well of a multi well plate. One ml of PBMC suspension, at 105/ml, added to each well and incubated at 37°C for 2 h. RPMI 1640 medium was removed and PBMCs were washed again with RPMI 1640 medium. One ml RPMI medium was added to each well and incubated for 2 h at 37°C. One hundred ìl yeast suspension was added to per plate and plates were incubated for 1 h at 37°C in a 5% CO_2 humidified incubator. PBMCs were washed twice gently with RPMI 1640 medium and 1 ml 1% w/v tannic acid solution was added. Then wash with RPMI 1640 medium. Cover slip was covered with a drop of heat-inactivated FBS and dried in air. Cells were stained with May-Grünwald freshly diluted 1: 2 with buffer, for 5 min then cover slips were rinsed in buffer. PBMCs were stained with Giemsa solution, freshly diluted with buffer, for 15 min and rinsed in buffer. At last the cover slips were inverted on microscope slides and observed at 100X magnification (Nikon microscope, Japan). On each cover slip PBMCs were counted until at least 100 PBMCs were scored. At access phagocytosis, the number of ingested yeast particles per counted PBMCs was determined [7, 8].

Immunoglobulin Content: Serum immunoglobulin (IgG, IgM and IgA) concentrations were measured by ELISA technique (Microplate Reader® - DAS) using commercial test kit for IgA, IgG and IgM (MCA630®, Serotec), according to product description.

Statistical Analysis: Results expressed as mean±SEM Multiple comparisons were performed by ANOVA and followed by the Tukey honestly significant difference (HSD) test. In all analyses, the level of significance was set to ($P<0.05$ or 0.01).

RESULTS

Analysis of Dietary Mycotoxins: According to HPLC method, Deoxynivalenol (DON) was found to be the major contaminant. Zearalenone and 15-acetyl DON, which were found above detectable limits (0.2 mg/kg) in dietary ingredients, did not exceed detectable limits in diet (0.5 mg/kg), because, just 12.2% of diet, was corn. Contaminated diet contained 3.6 mg/kg of DON and control diet contained 0.4 mg/kg (Table 1).

Table 1: Mycotoxins content of experimental diets (mg/kg) according to HPLC analysis.

Mycotoxin	Corn	Wheat	Control diet	Contaminated diet
DON	7.3	2.6	0.4	3.6
Zearalenone	0.8	-	-	-
15-acetyl DON	0.5	-	-	-

Table 2: The effect of fusarium contaminated diet on phagocytosis percentage from day 7 to day 42. The values are presented as mean±SEM.

	Day					
Group	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Normal diet	52±5.32	54±3.83	52±7.78	51±5.31	53±8.81	51±4.81
Contaminated diet	54±2.74^{a}	53±8.33^{a}	44±4.62^{b}	42±7.13^{bc}	38±3.64^{c}	37±7.24^{c}

a-c values with different superscripts in each column differ significantly (P<0.05).

Table 3: Effect of fusarium contaminated diet on plasma IgM, IgG and IgA levels. The values are presented as mean±SEM.

Ig	Day	Control + Vaccine	Contaminated diet + Vaccine
IgM	7	134.5±13.3	138.6±32.5
	21	172.7±23.6	160.6±3.1
	42	155.2±36.3	147.3±2.2
IgG	7	1032.3±41.5	1052.4±9.8
	21	1567.6±34.7^{a}	1073.9±10.7^{b}
	42	1148.3±9.9	1098.5±11.3
IgA	7	33.65±7.4	32.74±6.6
	21	40.53±5.4	43.93±6.2
	42	38.66±3.9	40.45±8.4

a-b values with different superscripts differ significantly (P<0.05).

Phagocytosis Assay: The effect of fusarium contaminated diet on phagocytosis is presented in Table 2. On day 7 and 14 of the study, there was no statistically significant difference in phagocytosis of experimental groups. The comparison of phagocytosis percentage of contaminated diet group with control, from day 21 to 42, showed significantly ($P \leq 0.05$) lower percentage of phagocytosis. The lowest phagocytosis percentage was observed at days 35 and 42.

Immunoglobulin Content: According to Table 3, serum IgM concentrations decreased insignificantly (P=0.364) in mold contained diet group on days 21 to 42 when compared to vaccine control. Serum IgG concentrations in mold contained diet decreased significantly only on day 21 (P=0.031), whereas its levels did not altered significantly on day 7 and 42 (P=0.183). But, there were no significant changes in serum IgA concentrations, during the study.

DISCUSSION

The effects of several mycotoxins on the immune responses have been mainly confined to laboratory animal studies. A smaller pool of data exists where farm animals and cells derived from livestock species have been employed to evaluate the immune-toxicity of mycotoxins [9].

Experimental mycotoxicosis have been studied primarily using 3 sources of mycotoxins, namely chemically purified mycotoxins, fungal culture materials and feedstuffs naturally contaminated with mycotoxins. Purified mycotoxins are useful in studying the metabolism and mechanism of action of individual mycotoxins. Fungal culture material is economical to produce in the laboratory but may contain multiple unknown mycotoxins. Naturally contaminated feedstuffs contain mixtures of mycotoxins, which also will contain unidentified mycotoxins, but represent most closely the real dietary situation that is present on the farm. Mycotoxins in combinations may exert a greater negative health impact than the sum of their individual effects [10].

In present study the potential effects of fusarium contaminated diet on phagocytosis as an index of innate immune response and antibody titre as an index of acquired immune response were evaluated. According to present study phagocytosis was reduced in rabbits fed the contaminated diet. Phagocytes play an important role in nonspecific immunity. The results of the current study suggest an immunotoxic effect of Fusarium mycotoxin contaminated feed. This finding is in agreement with many studies of the effects of trichothecenes on different phagocyte activities. Mann *et al*. [11] observed a reduction in neutrophil function *in vivo* in T-2 toxin treated calves. Kidd *et al*. [12] observed a reduction in turkey macrophage adherence and phagocytosis

after exposure to DON *in vitro*. Takayama *et al*. [13] investigated the effect of DON on bovine neutrophils *in vitro* and found suppression of chemiluminescence. Pang *et al*. [14] noted that inhalation of T-2 toxin in swine causes depressed alveolar macrophage phagocytosis. Gerberick and Sorenson [15] observed reduced alveolar macrophage function *in vitro* when exposed to T-2 toxin *in vitro*.

Some information has been published on the possible immunomodulatory effects of feeding Fusarium mycotoxin- contaminated grains to domestic animals. Johnson *et al*. [16] fed 36 to 44 ppm of DON to horses and found no significant effects of diet on serum IgA. Serum IgM concentrations were not measured. These results are in agreement with the current experiment. Increased serum IgA and IgM concentrations were found in pigs fed the same blend of contaminated grains as the current study [17].

Ingested DON affects intestinal Ig synthesis. Specifically, DON stimulates intestinal IgA production in mice, leading to an elevated concentration of circulating serum IgA [18, 19]. But in the present study fusarium contaminated diet caused significant reduction in IgG levels. This reduction could be related to cytotoxic effects of fusarium toxins on lymphocytes too.

In conclusion, in the current study the detrimental effects of dietary fusarium contaminated diet on immuno-responses suggest that an accurate monitoring system for mycotoxin contamination in feeds is desirable for assurance of poultry health and welfare.

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