

IMMUNOTOXICITY OF OCHRATOXIN A AND CITRININ IN NEW ZEALAND WHITE RABBITS

Kumar M., Dwivedi P., Sharma A.K., Telang A.G., Patil R.D., Singh N.D.

Division of Pathology, Indian Veterinary Research Institute, IZATNAGAR-243 122, India.

ABSTRACT: In the present study, the effects of ochratoxin A (OTA), citrinin (CIT) and their combination on the immunological parameters were studied in 6-8 weeks old New Zealand White rabbits at 60 d post-intoxication. Thirty two rabbits were divided into four groups of eight. OTA, CIT and a combination of the two were given at 0.75 mg/kg, 15 mg/kg and 0.75 plus 15 mg/kg, in feed respectively for up to 60 d of the trial. Other fourth group was used as a control, being fed standard toxin free feed. The toxin treated animals showed a significant decline in antibody titres to sheep red blood cells. The reduction in the cell mediated immune response was more significant in the OTA and combination groups as observed in the skin hypersensitivity test and the lymphocyte proliferation assay. The citrinin-treated rabbits failed to show any significant changes following the lymphocyte transformation assay and the delayed type hypersensitivity test. Histologically, the cellular (mononuclear cells) reaction in the skin in the treated groups was comparatively lesser than that of the control group. Thus, the present study in rabbits indicated significantly lower humoral and cellular immune responses in the OTA and combination groups

Key words: Citrinin, ochratoxin A, immunotoxicity, lymphocyte proliferation assay.

INTRODUCTION

Fungal contamination of feedstuffs and feed is one of the central problems in farming as this can reduce the nutritional value of feedstuffs and feed and is responsible for causing mycotoxicosis (CAST, 1989). The immunosuppressive properties of mycotoxins lead to an increased susceptibility to infections and vaccination failure, which in turn, results in heavy economic losses for livestock industries (Vasanthi and Bhat, 1998). Ochratoxin A (OTA) and citrinin (CIT) are key mycotoxins which can cause nephrotoxicity in different animals. OTA is mainly produced by *Aspergillus ochraceus* and CIT is produced by *Penicillium citrinum*. Allegedly OTA induces nephrotoxicity, immunotoxicity, genotoxicity, teratogenicity and carcinogenicity. The clinico-pathological changes include hypoproteinemia, poor growth, enhanced clotting time and widespread hemorrhages (Arai and Hibino, 1989). CIT is primarily a nephrotoxic compound which is often encountered together with OTA as a feed contaminant; however the level of acute toxicity varies in different species. In mice, CIT is embryocidal and fetotoxic, whereas in rats, at high doses, it causes teratogenic effects, renal tumors and induces the enlargement of and tubular necrosis of the kidney (Mayura *et al.*, 1984; Arai and Hibino, 1989). CIT together with OTA has been reported as an aetiological agent in the fatal human kidney disease - Balkan endemic nephropathy. CIT can act synergistically with OTA to depress RNA synthesis in murine kidneys (Sansing *et al.*, 1976). Although both OTA and CIT are nephrotoxic and have been reported to occur naturally as co-contaminants (Manickam *et al.*, 1985), studies involving OTA and CIT interaction are limited and no report is currently available regarding the interaction of these mycotoxins in rabbits. In India, natural outbreaks with heavy mortality due to ochratoxin contamination and their residual effects were reported in rabbits (Sharma,

1998; Prabu, 2004). As the rabbit is highly susceptible to commonly occurring mycotoxicoses, it has been recommended by the regulatory bodies, as one of the most suitable laboratory animal models for various toxicological evaluations involved in the execution of risk assessment for both animal and human populations (Derelanko and Hollinger, 2002). Therefore, the present work was undertaken to assess immunotoxicity in rabbits fed a diet containing low levels of OTA and CIT separately and in combination.

MATERIALS AND METHODS

Production and analysis of ochratoxin A

Pure culture of *Aspergillus ochraceus* (NRRL-3174) obtained from the National Center for Agricultural Utilization Research (NCAUR, Peoria, Illinois, USA) was employed for the OTA production as described by Trenk *et al.* (1971). OTA was produced through the fermentation of sterilized maize at $25 \pm 2^\circ\text{C}$ using the inoculated fungal culture for 2-3 weeks. OTA was extracted using chloroform and 10 g of diatomaceous earth (Celite-545) to 50 g of culture powder and then filtered. The filtrate was passed through a chromatography column containing activated diatomaceous earth and aqueous sodium bicarbonate. Initial elution was carried out with n-hexane, followed by benzene: acetic acid (98:2). Finally, OTA was estimated using thin layer chromatography (TLC) and a UV-Vis-Spectrophotometer (Genesys™ 10, Thermo Electron Corporation, Pittsford, USA) at 333 nm against the standard toxin procured from Sigma Chemicals Limited, USA (AOAC, 1995). The purity of the toxin was found to be approximately 94%.

Production and analysis of citrinin

CIT was produced and analyzed using sterile maize in accordance with the method described by Jackson and Ciegler (1978). The culture of *Penicillium citrinum* NRRL 5907, supplied by the National Center for Agricultural Utilization Research (NCAUR, Peoria, Illinois, USA), was used to produce CIT on partially ground maize at 25°C (± 2) for 2-3 weeks. The culture substrate was steamed for 5 min, dried at 50°C and ground to a fine powder. The culture was soaked with chloroform, acidified with concentrated HCl and allowed to equilibrate. The suspension was blended, filtered and the chloroform layer was rinsed with water. Next, the suspension was extracted with acidified 0.1 M NaHCO_3 (conc. HCl, pH 2.5) and condensed to $1/10$ volume. The crude chloroform extract was washed with water and again extracted using 0.1 M NaHCO_3 . The aqueous layer was acidified to pH 2.5, and toxin was collected by filtration. CIT was dissolved in chloroform and estimated using TLC and a UV-Vis-Spectrophotometer (Genesys™ 10, Thermo Electron Corporation, Pittsford, USA) at 322 nm against the standard CIT procured from Sigma Chemicals Limited, USA. The toxin was found to be approximately 95% pure.

Experimental procedure

Thirty two New Zealand White rabbits, 6-8 weeks of age, were obtained from the Laboratory Animal Resource Section of the Indian Veterinary Research Institute (IVRI) and were housed individually in metal cages. All the experimental procedures were conducted with the prior permission of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC). All animals were house-caged in a temperature-controlled and artificially illuminated room (12 h light/dark cycles). The rabbits were maintained on a standard basal diet supplied by the Feed Processing Unit of the Institute. Prior to commencing the research, the basal experimental diet was tested in the laboratory for aflatoxin B_1 , OTA, CIT and fumonisin B_1 . No detectable levels of these mycotoxins were observed in TLC and spectrophotometry analysis (lowest detection limit 1 to 5 $\mu\text{g}/\text{kg}$ feed). Fresh green fodder was provided along with water *ad libitum*.

After a one week of acclimatization period, the animals were randomly distributed into four groups of eight and treated as follows: Group I was given a diet containing 0.75 mg OTA/kg feed; Group II, a diet containing 15 mg CIT/kg feed; Group III: a diet containing 0.75 mg OTA/kg feed and 15 mg CIT/kg feed; and finally, Group IV was fed a standard mycotoxin free basal diet.

Immunological studies

Humoral immune response: On day 40 of the experiment, four rabbits, one from each group, were immunized with sheep red blood cells (SRBCs) by intraperitoneal injection of 0.5 mL of SRBCs suspended in phosphate buffer saline (PBS) at 1.25×10^6 cells per animal. After 10 d, following initial sensitization with SRBCs, the rabbits were given a booster dose of SRBCs. Serum was collected 10 days after the booster dose. A haemagglutination (HA) test was carried out using the micro-titration technique according to the procedure described by Beard (1980). The HA pattern (a diffuse sheet (lattice) of agglutinating RBCs covering the bottom of the wells) was noted and the titer was regarded as the reciprocal of the highest dilution, displaying complete agglutination of erythrocytes and expressed as $\log_2/0.05$ mL.

Cell mediated immune response

Lymphocyte proliferation assay: A lymphocyte proliferation test using MTT (3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide) was performed on day 60 of the trial. The procedure described by Bounous *et al.* (1992) was followed, although with some modifications.

Isolation of blood mononuclear cells: Blood samples (3-4 mL) were drawn aseptically with ethylenediamine tetra-acetic acid (EDTA) (0.5 mg/mL) as an anticoagulant. Following centrifugation, the plasma was removed and a buffy coat was collected and resuspended in 3 mL of PBS. This was then carefully layered over 2 mL of histopaque (1.077 g/mL, Sigma) and the tubes were centrifuged at 2000 rpm for 45 min. Leucocytes at the histopaque-PBS interface were carefully aspirated and rinsed three times with PBS. The resulting cells were suspended in a sufficient volume of RPMI-GM to give the required viable cell concentration to ensure that the next stages could be carried out. The cells were adjusted to give 5×10^6 cells/mL in media. 100 μ L of the cell suspension was added to 3 sets of triplicate wells of RPMI-GM growth medium (Roswell Park Memorial Institute - Granulocyte Macrophage medium). The second and third sets received 100 μ L of RPMI-GM containing 100 μ g/mL of Concanavalin-A. The plates were incubated at 37°C in a humidified chamber at 5% CO₂ tension for 96 h. Following this, 100 μ L of supernatant was removed from the wells and 10 μ L of MTT solution was added to all the wells. The plate was incubated for a further 4 h. MTT formazan was extracted from the cells using dimethyl-sulphoxide (100 μ L/well). OD was measured using an ELISA reader at a test wavelength of 492 nm and a reference wavelength of 650 nm.

Delayed type hypersensitivity response: Four rabbits from each group, were assessed for the effects of the various treatments on cell mediated immune response. On day 40 of the trial, the rabbits were injected intraperitoneally with 1 mg of ovalbumin in 1 mL PBS to assess sensitization. The right ear sites were challenged intradermally with 50 μ g ovalbumin in 50 μ L PBS after 10-15 d post-sensitization. The left ear sites (as negative controls) were injected with PBS only. The thicknesses of the ear sites were quantitatively measured using electronic digital calipers (Forbes-150 \times 0.01mm/6 \times 0.005) at 0, 24, 48, 72 h after challenge. At each interval, skin biopsies were collected under local anesthesia. These were preserved in neutral buffered formalin for histopathological evaluation.

All the data pertaining to the immunological findings were statistically analyzed using analysis of variance (ANOVA). The Student's post hoc t-test was applied to assess the level of significance among the groups (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

The rabbit is a species which is sensitive to OTA toxicity (median lethal dose: LD₅₀ 10 mg/kg of body weight (BW)) (Mir *et al.*, 1999) in comparison with other laboratory animals viz. rat and mice (LD₅₀ 20-30 mg/kg BW and 46-58 mg/kg BW, respectively) (Marquardt and Frohlich, 1992). The highest concentrations recorded, up to 80 ppm OTA and 9.6 ppm ochratoxin B (OTB), were reported in Italy, where these were found in mouldy bread (Visconti and Bottalico, 1983). Exposure to this caused lethal gastroenteritis in rabbits, chicken and dogs. An outbreak of ochratoxicosis in angora rabbits, resulting in a heavy mortality rate, was also reported from Himachal Pradesh, India (Sharma, 1998). Hence, in the present study, the rabbit was selected as the experimental animal for the assessment of OTA- and CIT-induced immunotoxicity.

Humoral immune response

The humoral immune response was assessed by measuring the antibody titres against SRBCs ($\log_2/0.05$) in all the groups. There was a significant reduction in the \log_2 values for HA titres in the sera of groups I (3.75 ± 0.48), II (2.75 ± 0.25) and III (3.50 ± 0.29) rabbits when compared to that of group IV (5.50 ± 0.29) at day 60 of the study (Table 1). The decrease in HA titres may be due to the depletion of lymphocytes from the lymphoid organs as observed histopathologically in the spleen and Peyer's patches (unpublished data), since SRBCs were thymus-dependent antigens and required T and B cell co-operation for antibody synthesis (Toivanen *et al.*, 1972). Several studies revealed that OTA affected the structural components of the immune system. Immune suppression due to regression of immunological organs such as the bursa of Fabricius and the thymus and their lymphoid cell population in the OTA treated chicks, resulted in a decline in IgG, IgA and IgM levels in serum (Dwivedi and Burns, 1985). This result lends further support to the present findings. In the CIT group antibody titres to SRBCs were also reduced suggesting humoral immunosuppression. A significant decrease was observed in the relative lymphocyte counts in treated groups I, II and III (51.75%, 52.50% and 51.00%) compared to control group IV (64.75%) at day 60 of the trial (Kumar *et al.*, 2007). The present findings in CIT-treated animals contrasts with previous studies which reported either no effect on humoral immune response (Campbell *et al.*, 1981) or an increased humoral immune response (Reddy *et al.*, 1988) in CIT toxicosis. Regarding the animals in the combination group, the reduction in antibody titres could be attributed to the immunotoxic effect of both types of toxin.

Cell mediated immune response

Concanavalin A induced stimulation indices of the lymphocytes collected from rabbits from all the groups at day 60 of the trial, were significantly lower in groups I and III when compared to those of the control group. Group III (0.81 ± 0.01) animals revealed the lowest stimulation index, followed by group I (0.83 ± 0.01) and group II (0.97 ± 0.01). Group IV control rabbits displayed the highest stimulation index (1.08 ± 0.03) (Table 1).

In general, the ear pinna revealed hot and painful focal swelling at 24 h (Groups I, II, III and IV, 0.18, 0.19, 0.17 and 0.20 mm, respectively) which tended to decrease after 48 h (Groups I, II, III and IV, 0.16, 0.17, 0.15 and 0.18 mm, respectively) and 72 h (Groups I, II, III and IV, 0.13, 0.14, 0.12 and 0.15 mm, respectively) in rabbits from all four groups. The increase in skin thickness was relatively lower in the intoxicated groups when compared with that of the control group. Histopathological examination of skin biopsies collected from the control group rabbits revealed varying degrees of acute inflammation characterized by severe congestion of blood vessels together with hemorrhages, oedema and mononuclear cell infiltration. The dermis was chiefly affected showing intense infiltration especially by mononuclear cells combined with a few lymphocytes and heterophils. In comparison, the epidermis remained

Table 1: Means and standard error (mean±SE) of the effects of OTA, CIT and their combination on humoral and cell mediated immune responses in rabbits, measured 60 d post intoxication (n=4).

	Group I (OTA)	Group II (CIT)	Group III (OTA+CIT)	Group IV (Control)
HA (log ₂) ¹	3.75±0.48 ^b	2.75±0.25 ^c	3.50±0.29 ^{bc}	5.50±0.29 ^a
S.I. ²	0.83±0.01 ^b	0.97±0.01 ^a	0.81±0.01 ^b	1.08±0.03 ^a

¹HA (log₂): haemagglutination titre against SRBCs (sheep RBC) in terms of log₂/0.05 mL serum.

²S.I.: Stimulation index (Lymphocyte transformation assay).

Means within a column with different superscripts differ at $P < 0.05$, using Student's 't' test as a post hoc test.

unaffected. Skin biopsies, taken from groups I and III and collected at various intervals, revealed a lower level of cellular reaction when compared to that of the control group. However, in contrast to the other two intoxicated groups, the CIT-treated animals displayed a greater degree of cellular reaction which was comparable to that observed in the control group (Figures 1-4).

Reductions in the cell-mediated immune responses, in the OTA and combination groups, as assessed by the delayed type hypersensitivity (DTH) response and lymphocyte proliferation assay, were supported by earlier observations in OTA treated poultry (Dwivedi, 1984; Singh, 1990; Ramadevi *et al.*, 1996), pigs (Harvey *et al.*, 1992) and rats (Satheesh *et al.*, 2005), where significant cellular immunosuppression was observed. The stimulation index of the lymphocyte transformation test in rabbits indicated a significantly lower cellular immune response in OTA and combination groups. This may be due to the effect of these toxins on the immunocompetent organs (Chang, 1979; Dwivedi and Burns, 1985). These findings were further supported by a reduced cellular reaction in the dermis at the skin test site, as histopathologically noted. The cell mediated immune response was not affected by CIT. This observation is supported by earlier reports (Campbell *et al.*, 1981; Reddy *et al.*, 1988). In conclusion, OTA alone and also in combination with CIT caused severe humoral and cellular immunodepression in rabbits. Furthermore, the interaction between these two mycotoxins appears to be more severe and additive in nature. However, in CIT-treated animals the effect on cell mediated immunity was minimal, although it did result in the depression of the humoral immune response.

Acknowledgements: The authors would like to acknowledge the help extended by the Director of the IVRI and the Head of the Division of Pathology in the execution of this research. This study was financially supported in part by the NATP-CGP (ICAR) Project No. II/221.

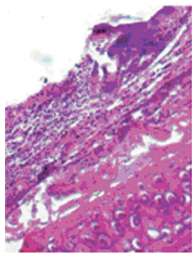


Figure 1: Skin delayed type hypersensitivity response (Group I (OTA), 48 h): the epithelium is hyperplastic and thickened showing a reduced number of inflammatory cells in the dermis and also mild oedema. H&E×100.

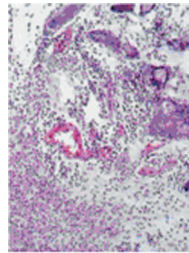


Figure 2: Skin delayed type hypersensitivity response (Group II (CIT), 24 h): the dermis showing oedema, diffuse infiltration of inflammatory cells consisting of mononuclear cells and polymorphs, together with severe engorgement of the blood vessels. H&E×100.

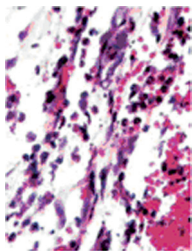


Figure 3: Skin delayed type hypersensitivity response (Group III (OTA+ CIT), 24 h): the dermis showing decreased cellular infiltration (minimum) with moderate engorgement of the blood vessels and oedema. H&E×400.

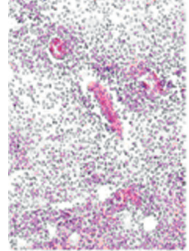


Figure 4: Skin delayed type hypersensitivity response (Group IV (Control), 24 h): the dermis showing oedema and diffuse heavy infiltration of inflammatory cells consisting of mononuclear cells and polymorphs, together with severe engorgement of the blood vessels. H&E×100.

REFERENCES

- AOAC. 1995. Official Method of Analysis. (16th ed.), Association of Official Analytical Chemist Arlington, VA, USA.
- Arai M., Hibino T. 1989. Review of mycotoxin contamination. *J. Assoc. off. Anal. Chem.*, 72: 223-230.
- Beard C.W. 1980. Serological procedure, isolation and identification of avian pathogens. (2nd ed.) Creative Printing Company, New York. pp: 129-135.
- Bounous D.I., Campagnoli R.P., Brown J. 1992. Comparison of MTT colorimetric assay and tritiated thymidine uptake to lymphocyte proliferation assays using chicken splenocytes. *Avian Dis.*, 36: 1022-1027.
- Campbell M.L., Doerr J.A., Wyatt R.D. 1981. Immune status in broiler chickens during citrinin toxicosis. *Poult. Sci.*, 60: 1634.
- Chang C.F., Huff W.E., Hamilton P.B. 1979. A leucocytopenia induced in chicken by dietary ochratoxin A. *Poult. Sci.*, 58: 555-558.
- Council for Agricultural Science and Technology. 1989. Mycotoxins: Economic and Health Risks. Task Force Report. No116, Ames IA, Iowa.
- Derelenko J.M. Hollinger, A.M., 2002. Handbook of Toxicology, (2nd ed) CRC press LLC, 2000 N. W. Corporate Blvd., Boca Raton, Florida 33431
- Dwivedi P. 1984. The immunological and pathological changes in poultry induced by ochratoxin A. *Ph. D thesis. Univ. of Edinburgh (UK)*.
- Dwivedi P., Burns R.B. 1985. Immunosuppressive effects of ochratoxin A in young turkeys. *Avian. Pathol.*, 14: 213-215.
- Harvey R.B., Elissalde M.H., Kubena L.F., Weaner E.A., Corrier D.E., Clement B.A. 1992. Immunotoxicity of ochratoxin A to growing gilts. *Am. J. Vet. Res.*, 53: 1466-1470.
- Jackson L.K., Ciegler A. 1978. Production and analysis of citrinin in corn. *Appl. Environ. Microbiol.*, 36: 408-411.
- Kumar M., Dwivedi P., Sharma A K., Telang A G., Patil R D., Singh N. D. 2007. Clinical and haematological alterations induced by ochratoxin A, citrinin alone and in combination in young growing rabbits. *Indian J. Vet. Pathol.*, 31: 45-49.
- Manickam A., Palaniswami A., Neelakantan S. 1985. Mycotoxicosis in buffaloes. *Indian Vet. J.*, 62: 711-714.
- Marquardt R.R., Frohlich A.A. 1992. A review of recent advances in understanding ochratoxicosis. *J Anim Sci.*, 70: 3968- 3988.
- Mayura K., Parker R., Berndt W.O., Phillips T.D. 1984. Effects of simultaneous prenatal exposure to ochratoxin A and citrinin in the rat. *J. Toxicol. Environ. Hlth.*, 13: 553 - 561.
- Mir M. S., Dwivedi P., Charan K. 1999. Ochratoxin A induced acute toxicity in rabbits. *Indian J. Vet. Pathol.*, 23: 8-13.
- Prabu P.C. 2004. Pathological studies on experimental aflatoxicosis and ochratoxicosis and their interaction in rabbits. MVSc. Thesis submitted to Deemed University, IVRI, Izatnagar, U.P, India.
- Ramadevi V., Naidu N.R.G., Rao M.V.S. 1996. Effect of ochratoxin A on immune system in broiler chickens. *Indian Vet. J.*, 73: 722-724.
- Reddy R.V., Taylor M. J., Sharma R. P. 1988. Evaluation of citrinin toxicity on the immune functions of mice. *J. Fd. Prot.*, 51: 32-36.
- Sansing G.A., Lillehoj E.B., Detroy R.W. 1976. Synergistic toxic effect of citrinin, ochratoxin A and penicillic acid in mice. *Toxicon*, 14: 213-220.
- Satheesh C.C., Sharma A.K., Dwivedi P., Prasanna K., Patil R.D., Rahul. S. 2005. Immunosuppressive effect of ochratoxin A in rats. *J. Ani. Vet. Adv.*, 4: 603-609.
- Sharma, P. (1998). Himachal Pradesh rabbit farmers fear toxin in feed: Mortality, infertility cases on rise. *The Hindustan Times, New Delhi, Thursday, July 30th 1998. Vol. LXXIV No. 210, p. 3.*
- Singh G.S.P., Chauhan H.V.S., Jha G.J., Singh K.K. 1990. Immunosuppression due to chronic ochratoxicosis in broiler chicks. *J. Comp. Pathol.*, 103: 399-410
- Snedecor G.W., Cochran W.G. 1989. Statistical Methods. (8th ed). Iowa State Univ. Press, Ames.
- Toivanen P., Toivanen A., Good R.A. 1972. Ontogeny of bursal function in chickens. I. Embryonic stem cell for humoral immunity. *J. Immunol.*, 109: 1058-1070.
- Trenk H.L., Butz M.E., Chu F.S. 1971. Production of ochratoxin in different cereal products by *Aspergillus ochraceus*. *Appl. Microbiol.*, 21: 1032-1035.
- Vasanthi S., Bhat R.V. 1998. Mycotoxins in food - occurrence, health and economic significance and food control measures. *Indian J. Med. Res.*, 105: 212-224.
- Visconti A., Botalico A. 1983. High levels of ochratoxins A and B in mouldy bread responsible for mycotoxicosis in farm animals. *J. Agric. Fd. Chem.*, 31, 1122-1123.