

Immunosuppressive Effect of Ochratoxin A in Wistar Rats

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Abstract: Ochratoxins are potent mycotoxins elaborated by *Aspergillus* and *Penicillium* species. Incidence of ochratoxin A (OT-A) in feed and natural occurrence of ochratoxicosis in animals have been reported from various parts of the world, including India. In this study, we report the immunosuppressive effect of ochratoxin A in rats. The cell mediated immune responses, as assessed by 2, 4-di-nitro-chloro-benzene (DNCB) induced contact hypersensitivity reaction and macrophage function test (NO_2^- production assay) were found to be decreased significantly in OT-A intoxicated rats as compared with those in controls. Humoral immune response, assessed by antibody titres against sheep red blood cells, was also found to be decreased in toxin fed rats. The haemato-biochemical alterations such as leucopenia, lymphopenia and hypoproteinemia associated with OT-A toxicity were also indicative of immunosuppression. These findings suggest that OT-A induces the suppression of both humoral and cell mediated immune responses and must be taken into account while dealing with cases of vaccination failure or frequent bacterial and viral infections in livestock and poultry.

Key words: Ochratoxin A, immunosuppression, wistar rats, macrophage function assay

Introduction

Ochratoxins, potent mycotoxins, are dihydroisocoumarin metabolites produced by several species of *Aspergillus* and *Penicillium* (Van der Merwe *et al.*, 1965 and Marquardt and Frohlich, 1992) Experimental studies have revealed ochratoxin A (OT-A) to be toxic to several species of animals tested, including mice (Carlton *et al.*, 1968; Budiarmo *et al.*, 1971), rats, (Kanisawa *et al.*, 1977; Thuvander *et al.*, 1996 and Wangikar, 2004) and guinea pigs (Carlton and Tuite, 1970), swine (Szczzech *et al.*, 1973a), dog (Szczzech *et al.*, 1973b), weaned calves (Pier *et al.*, 1976) and duckling and chicks (Dwivedi, 1984). The depletion of lymphoid cells after OT-A ingestion, particularly in the thymus, bursa of Fabricius, spleen and Peyer's patches has been reported in different species of poultry (Dwivedi and Burns, 1984a, 1985). In poultry, diminution in size and depletion of lymphoid cells in thymus occurred concurrently with a reduced delayed hypersensitivity response and decreased graft versus host reaction, suggesting a severe cellular immunosuppression by OT-A besides suppression of humoral immune response (Dwivedi and Burns, 1984b). Although the immunosuppressive effects of ochratoxins have already been reported in different species, a systematic effect of subchronic toxicity of OT-A on immune response in a mammalian model has not been carried out. Moreover, the effect of this toxin on macrophage activity in terms of nitric acid production on stimulation has not been reported earlier. Hence, the present study was aimed to evaluate the immunotoxic effect of ochratoxin A in rats using several immunological assays.

Materials and methods

Production of ochratoxin A (OT-A) and preparation of toxin contaminated feed: OT-A was produced by growing *Aspergillus ochraceus*, NRRL-3174, procured from National Centre for Agriculture Utilization Research (NCAUR), Peoria, Illinois, U.S.A. on sterile maize as per the method described by Trenk *et al.* (1971). Cultured maize powder containing 100 ppm of ochratoxin A was added to the basal rations (which were tested negative for the presence of contaminating mycotoxins) in such proportion that the final concentration of OT-A was 4 ppm. Aliquots were taken from the mixed diet and toxin was quantified to ensure the proper mixing of concentrated toxin in cultured maize.

Experimental animals: A total of sixty male Wistar rats, 5-7 weeks of age, procured from Laboratory Animal Research Section, IVRI, Izatnagar, were maintained on a formulated mycotoxin free feed with *ad libitum* water. The animals were divided into two groups (each group was subdivided so as to have four animals for cell mediated immune studies, four for humoral immune studies, six for macrophages function studies and 16 for haematobiochemical studies) of which one group was fed with the toxin (4 ppm) containing feed while the other group was kept as control. The experimental procedures conducted on the experimental animals were duly approved by the Institute's Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Immunological studies:

Cell mediated immune response (DNCB induced cutaneous contact sensitivity reaction): Four rats from each group were assessed for the effect of ochratoxin-A on cell mediated immune response. The skin site on the abdominal wall was prepared by shaving and gently rubbing with cotton swab soaked with 70% alcohol. An area of 3 cm² was marked. The animals were sensitized with 0.5% 2, 4-dinitro-chloro-benzene (DNCB) (Sigma) in 4% acetone:olive oil on 30th day of experiment by two daily topical applications of two drops (0.04 ml) and was spread with a glass rod. Control animals were applied vehicles in similar manner.

Ear site challenge: The initial thicknesses of both ears of all the rats were recorded after 9 days of sensitization using electronic digital calipers (Forbes-150x0.01 mm/6x0.005). Following the measurement, rats were challenged on dorsal side of each ear lobe with one drop of 0.2% DNCB in the same vehicle. Thicknesses of both the ears were re-measured at 24, 48 and 72 h post challenge. The challenged area of the skin was examined for development of gross lesions (erythema, induration, vesicle or scab formation). Skin biopsies from the ears were taken at all intervals and preserved in 10% formal saline for histopathological studies.

Humoral immune response (Haemagglutination test): Four rats from each group were immunized by intraperitoneal injection of 0.25 ml suspension of with sheep red blood cells (SRBCs) in phosphate buffered saline (PBS) (1.25×10^6 cells/rat) on 30th day of the experiment. Four control rats received intraperitoneal injection of sterile PBS. The animals were challenged after 10 days. Serum was collected after 6 days of challenge. Haemagglutination (HA) was carried out by microtitration techniques according to the procedure described by Beard (1980) with slight modification. Briefly, HA test was performed in U shaped micropersplex plate. Two-fold serial dilution of serum was prepared in PBS keeping the final volume of 0.05 ml in each well except in control well, which contained PBS alone. Then 0.05 ml of 0.5% SRBC suspension was added to all the wells. A known negative control was also included. The plate was swirled gently for mixing and uniform distribution of erythrocytes and left at room temperature for 40-45 minutes. The HA pattern (a diffused sheet of agglutinating RBC covering the bottom of the wells) was read with the aid of a hand lens and the titre was recorded as reciprocal of the highest dilution showing complete agglutination of erythrocytes and expressed as $\log_2/0.05$ ml.

Macrophage function assay: Six animals each from the toxin fed and control groups were subjected to macrophage function assays. Animals on 30th day of experiment were euthanised. The abdomen was swabbed with 70% alcohol to sterilize the area. A midline incision was made with a sterile scissor. Then the abdominal skin was retracted with forceps to expose the intact peritoneal wall. The peritoneal cavity was filled with harvest medium using a 30 cc syringe attached with a 19-G needle and massaged gently. The peritoneal lavage was retrieved and viable cells were counted by trypan blue dye exclusion method. The peritoneal macrophages were suspended in phenol red free RPMI-1640 (Sigma) growth medium containing 5 mM L - arginine, at a concentration of 1×10^6 cells/ml. One hundred microlitre of this suspension was put into each well of 96 wells flat bottom culture plate in triplicate. After 2 h of incubation, the non-adherent cells were removed by washing with warm PBS (37°C). The final volume of wells was made to 200 μ L with *Escherichia coli* Lipopolysaccharide (LPS) (Sigma) which was added at a concentration of 1 mg ml⁻¹. The plates were incubated at 37°C in a humidified chamber containing 5% CO₂. Culture supernatants were taken at 24, 48 and 72 h interval and were assayed for nitrite production by Griess reagent. 50 μ L culture supernatant was incubated with equal amount of Griess reagent (1% sulphanilamide and 0.1% N (1-naphthyl) ethylene diamine dihydrochloride) at room temperature for 10 min. After incubation, absorbance at 570 nm was recorded and nitrite was quantified from graph prepared using sodium nitrite as standard (Green *et al.*, 1982).

Haemato-biochemical observations: Blood was collected from four animals of each group at fortnightly intervals by retro-orbital puncture. The total leucocyte count (TLC) and differential leucocyte counts (DLC) were carried out (Benjamin, 1985). The blood (1.5 -2 ml) was collected separately in a dry, clean and sterilized test tube and allowed to clot. The sera samples were preserved at -70°C till the estimations were carried out. The sera samples were analysed for the total protein (Biuret method) and albumin (BCG dye binding method) using standard kits (Qualigens Diagnostics). The values of globulin were derived by subtracting the values of albumin from that of total proteins. The data generated from haemato-biochemical observations was analyzed by using one way analysis of variance (ANOVA) and a probability of p=0.05 was accepted as significant (Snedecor and Cochran, 1995).

Results

Cell mediated immune response was evaluated employing delayed type contact sensitivity reaction to DNCB. The increase in skin thickness of ear pinna was measured at 24, 48 and 72 h post challenge with DNCB, in different groups. There was apparent decrease in skin thickness in the toxin fed group compared with that of control group,

at all intervals (Fig. 1) and a significant reduction could be detected at 72 h (0.08 vs 0.18 mm). The DNCB treated pinna revealed hot, painful, focal to diffuse swelling at 24 h, which in toxin fed groups decreased considerably by 48 to 72 h. In control group, however, the reaction was more severe with persistent swelling which revealed sloughing of superficial epithelium, forming scab at the later stage.

Histopathological examination of skin biopsies collected at 24, 48 and 72 h revealed varying degrees of acute inflammation characterised by extensive congestion with haemorrhage, oedema and neutrophil infiltration at 24 h. At 48 h, the intensity of inflammation in the toxin fed group was considerably reduced compared with those in control. In the control group, along with infiltration of neutrophils marked infiltration of macrophages was also seen at this stage (Plate 1 and 2). At 72 h, cutaneous reaction in the toxin fed group almost subsided except sparse infiltration of mononuclear cells and mild oedema into the dermis. However, in control group, the superficial necrotic epidermal cells formed the scab. The dermis showed infiltration of macrophages with fibroblastic proliferation and the detachment of cartilage with the adjoining tissue.

Humoral immune response: The humoral immune response as assessed by measuring the haemagglutination titre against sheep RBCs in terms of $\log_2/0.05\text{ml}$, showed significant decrease in toxin fed rats compared with the control ones (3.33 ± 0.33 vs 8.00 ± 0.58).

Macrophage function assay: The resident peritoneal macrophages were collected and cultured. The cell recovery (10^6 cells) and amount of nitrite produced per $50\mu\text{L}$ supernatant of resident macrophage culture were measured. Regarding the cell recovery, the values revealed significant differences in the toxin fed and control rats ($1.63 \pm 0.75 \times 10^6$ vs $2.63 \pm 0.80 \times 10^6$). The toxin fed group revealed a significant decrease ($2.52 \pm 0.19 \mu\text{mol} / 50\mu\text{L}$ of culture supernatant) in amount of induced nitrite produced at 72 h of culturing compared to that in controls ($3.10 \pm 0.16 \mu\text{mol} / 50\mu\text{L}$ of culture supernatant) (Fig. 2).

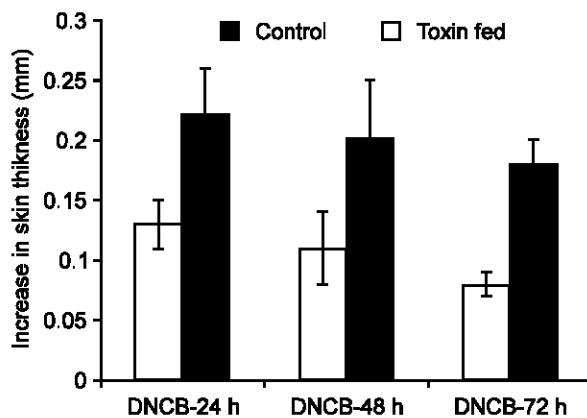


Fig. 1: Effect of OT-A on cell mediated immune response

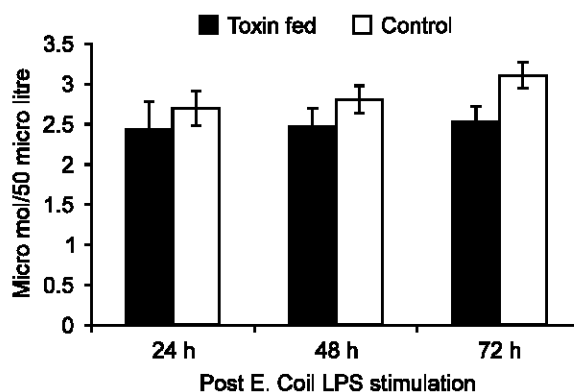


Fig. 2: Effect of OT-A on induced nitrite production (μmoles) by resident peritoneal macrophages

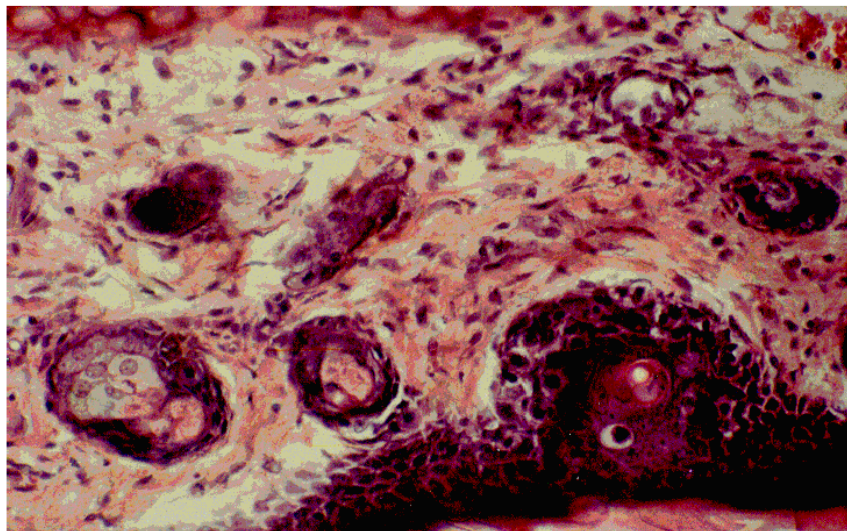


Plate 1: Skin (OT-A fed), Dermis showing sparse infiltration of mononuclear cells. Hand&E x 200

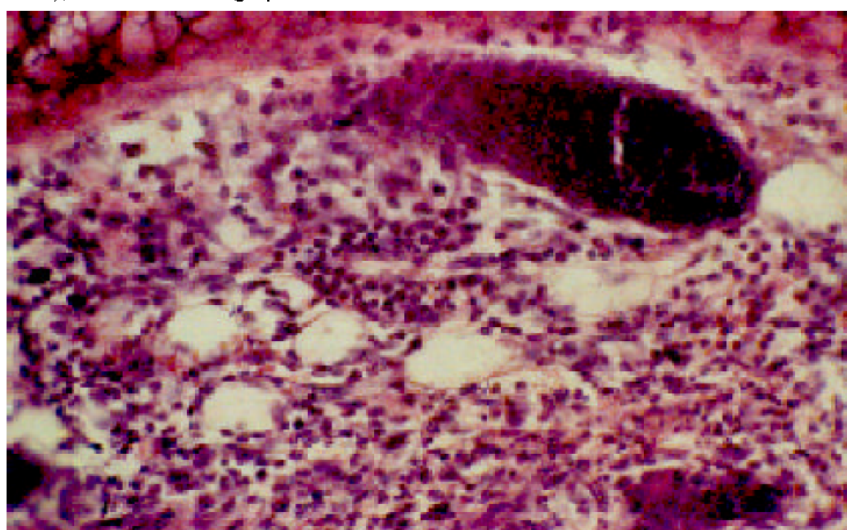


Plate 2: Skin (Control), Marked infiltration of polymorphs and macrophages in dermis. HandE x 200

Haematobiochemical changes: The data on haematobiochemical changes are presented in the Table 1. Total leucocyte counts showed a reduction in the toxin fed group when compared with that of the controls. The decrease was not significant at 2 and 4 weeks, while it was at 6 and 8 weeks compared with that in the control group. Absolute lymphocyte count revealed no significant differences among different groups at 2 and 4 weeks. However, significant decrease in lymphocyte count was observed in the toxin fed group at 6 (5.64 ± 0.54) and 8 weeks (5.34 ± 0.76) post treatment. A moderate relative increase in neutrophils in the toxin fed groups was noticed, which, however was not statistically significant. The absolute eosinophil, monocyte and basophil counts showed no significant differences among different groups.

Total protein values did not show significant differences among different groups at 2 and 4 weeks. However, there was significant reduction of total protein values at 6 and 8 weeks in the toxin fed group rats when compared with that of the control group.

The albumin values were reduced significantly after 6 weeks in the toxin fed groups (2.67 ± 0.54) compared with those of the control group (4.51 ± 0.36).

Albumin: Globulin ratio failed to reveal any significant differences at 5% level in any of the intervals other than at 6 weeks (1.20 ± 0.32) showed significant decrease compared with those of the control (2.19 ± 0.37).

Table I: Effect of OT-A on haemato-biochemical parameters (Mean± Standard Error) at different intervals in rats (Means bearing different superscript differ between groups, p=0.05)

	Interval	Toxin fed	Control
Total leucocyte count (thousands μL^{-1})	2 weeks	8.48±1.20	11.61±1.45
	4 weeks	10.14±0.94	9.99±0.81
	6 weeks	8.50±0.71 ^a	11.12±1.19 ^b
	8 weeks	8.63±0.97 ^a	12.78±0.98 ^b
Absolute lymphocyte count	2 weeks	6.25±0.70	8.23±1.32
	4 weeks	7.26±0.75	7.23±0.70
	6 weeks	5.64±0.54 ^a	8.51±0.82 ^b
	8 weeks	5.34±0.76	9.51±1.15
Absolute neutrophil count	2 weeks	1.52±0.40	2.65±0.17
	4 weeks	2.27±0.25	1.75±0.23
	6 weeks	1.96±0.32	1.59±0.27
	8 weeks	2.61±0.52	2.39±0.14
Total Protein (g dL^{-1})	2 weeks	5.26± 0.21	6.62±0.84
	4 weeks	5.31± 0.39	6.90± 0.83
	6 weeks	4.99±0.48 ^a	6.7± 0.52 ^b
	8 weeks	5.55± 0.34 ^a	7.25± 0.37 ^b
Albumin (g dL^{-1})	2 weeks	2.95±0.14	4.07±0.84
	4 weeks	3.13± 0.10	4.46± 0.83
	6 weeks	2.67± 0.54 ^a	4.51± 0.36 ^b
	8 weeks	2.93± 0.68	4.71± 0.42
Globulin (g dL^{-1})	2 weeks	2.31±0.10	2.56±0.31
	4 weeks	2.18± 0.30	2.44± 0.14
	6 weeks	2.32± 0.18	2.19± 0.32
	8 weeks	2.62± 0.38 ^a	2.53± 0.13 ^b
Albumin: Globulin Ratio	2 weeks	1.29±0.07	1.68±0.13
	4 weeks	1.50± 0.16	1.85± 0.38
	6 weeks	1.20± 0.32 ^a	2.19± 0.37 ^b
	8 weeks	1.12± 1.16	1.88± 0.22

Discussion

The cell mediated immune responses, as assessed by DNCB contact sensitivity reaction and macrophage function test (NO_2^- production) were found to be significantly decreased in OT-A intoxicated rats as compared with those in the control animals. These observations find support from earlier studies in poultry (Dwivedi, 1984; Singh *et al.*, 1990 and Ramadevi *et al.*, 1996) and pigs (Harvey *et al.*, 1992).

The humoral immune response was assessed by haemagglutination titres against SRBCs and was found to be significantly reduced in the toxin fed group. This was in accordance with the reports of Haubeck *et al.* (1981) who recorded a 50% reduction in IgM response to SRBCs in mice as a result of induced ochratoxicosis. Similar responses were also reported in poultry by earlier workers (Dwivedi, 1984; Dwivedi and Burns, 1984a, 1985).

The rats in the toxin fed group showed a significant reduction in macrophage cells recovery and nitrite production by resident peritoneal macrophages, indicating immunosuppression. Furthermore, the earlier reports of the depletion of mononuclear cells in ochratoxicosis (Thuvander *et al.*, 1995) authenticated the present findings. However, these findings are contradictory to those of Boorman *et al.* (1984) who observed a dose related increase in peritoneal macrophages in mice in ochratoxicosis. Though no reports are available regarding the effects of OT-A on nitrite production, various workers have reported immunosuppressive effects of OT-A on macrophages and mononuclear cells (Chang and Hamilton, 1980; Klinkert *et al.*, 1981; Chang, 1982; Singh *et al.*, 1990; Stormer and Lea, 1995 and Charoenpornsook *et al.*, 1998).

In haematological parameters, there was reduction in total leucocytic counts marked by lymphocytopenia with relative increase in the neutrophil percentage. Most of the literature available regarding the effects of OT-A on haematological

parameters are contradictory and inconsistent (Thacker and Carlton, 1977; Singh *et al.*, 1990; Harvey *et al.*, 1992 and Farshid and Rajan, 1993). Mild leucocytopenia observed in this study was essentially lymphocytopenic type, which could be associated with lymphoid depletion from lymphoid organs as observed histopathologically (Satheesh, 2003) and reduced cell mediated and humoral immune responses. Leucocytopenia associated with the lymphocytopenia has also been reported previously in rats (Wangikar, 2002), rabbits (Mir, 1998), guinea pigs (Thacker and Carlton, 1977), turkeys (Chang *et al.*, 1981), chicken (Singh *et al.*, 1990) and Japanese quail (Farshid and Rajan, 1995). Biochemical alterations in OT-A treated rats were decreased serum total proteins, albumin and globulins, which include α globulins (immunoglobulins) also. Possible reasons attributable to low levels of serum protein on OT-A toxicosis were anorexia (Satheesh, 2003), loss of proteins (albumin) in urine due to extensive damage to proximal convoluted tubular epithelial cells in kidneys (Prasanna, 2003) and in hepatocytes, the major site of protein synthesis (Satheesh, *et al.*, 2003; 2004). OT-A was found to inhibit the protein synthesis through competitive inhibition of phenylalanine t-RNA synthetase (Creppy *et al.*, 1979) in rats. Inadequate digestion and/or absorption due to OT-A induced damage in gastrointestinal tract might have also contributed to hypoproteinemia to some extent. The present study confirms that OT-A toxicosis caused significant immunosuppression due to reduction of both cell mediated and humoral immune status and should be taken in to consideration while dealing with problems of vaccination failures and increased host susceptibility to bacterial and viral infections in farm animals.

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