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PATHOLOGICAL STUDIES IN BROILER CHICKS FED AFLATOXIN OR OCHRATOXIN AND INOCULATED WITH INCLUSION BODY HEPATITIS VIRUS SINGLY AND IN CONCURRENCE

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ABSTRACT

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Day-old broiler chicks found negative for maternal antibodies against inclusion body hepatitis (IBH) virus by agar gel precipitation test and viral antigen in cloacal swabs by dot enzyme immunoassay were divided into 6 groups of 20 chicks each. Group A was fed aflatoxin B_1 at 1.25 ppm from 3 to 38 days of age; group O was fed ochratoxin A at 0.5 ppm from 3 to 38 days of age; group V was inoculated with 1 ml of IBH virus of titre $\log_{10}$ 6.5 EID_{50} per 0.2 ml. Groups AV and OV were given aflatoxin B_1 and ochratoxin A, respectively, and also infected with the virus. Group C served as control. There was mild enlargement and paleness of the liver up to 18 days post inoculation in group V; there were no lesions in group A; and there was gradual enlargement of the kidneys from 10 days post feeding of mycotoxin onwards in group O. In the combined groups AV and OV the gross lesions were slightly more severe. In group V, varying degrees of degenerative histopathological changes, congestion and haemorrhages were seen particularly in the liver, followed by the kidneys, bursa, spleen, myocardium and lungs, along with intranuclear inclusion bodies in the hepatocytes, mostly in the early stages of infection. Similar microscopic changes, but without inclusion bodies, were seen in groups A and O and the changes were pronounced in the later stages. In group O, the kidney lesions were more pronounced than the liver lesions. In the concurrently infected groups, AV and OV, the changes were similar but slightly more marked than in the corresponding individual groups. Inclusion bodies in hepatocytes were more frequent, more prominent and appeared earlier in the concurrent groups.

Keywords: aflatoxin, histopathology, inclusion body hepatitis virus, mycotoxin, ochratoxin, pathology

Abbreviations: AGPT, agar gel precipitation test; DPF, days post feeding of the mycotoxin; DPI, days post inoculation; EIA, enzyme immunoassay; EID_{50}, dose at which 50% of embryos will be infected; IBH, inclusion body hepatitis; PAU, Punjab Agricultural University

INTRODUCTION

Poor post-harvest management, storage in ill-ventilated stores, and an environment conducive for the growth of fungi result in a large proportion of the feed constituents, such as groundnut cake, maize, etc., destined for poultry consumption being contaminated with mycotoxins. Numerous episodes of mycotoxicosis have been recorded from the Punjab by Jassar (1986), by Raina and Singh (1991), and by Singh and colleagues (1994), particularly in young broiler chicks. Feed samples analysed at the Punjab Agricultural Laboratory revealed aflatoxins above the permissible level in

25%, 29%, 50% and 56% of 81, 110, 119 and 95 feed samples tested in 1986, 1987, 1989 and 1990, respectively (Jassar, 1986; Mangat *et al.*, 1987; Raina and Singh, 1991; Singh *et al.*, 1994). Likewise, Raina and Singh (1991) recorded ochratoxin in 10 out of 77 samples of poultry feed, while in 4 samples both ochratoxin and aflatoxin were present. Singh and colleagues (1994) observed ochratoxin and aflatoxin as co-contaminants in 3 out of 95 samples.

Inclusion body hepatitis (IBH) in broiler chicks aged 3–12 weeks typically causes hepatic lesions consisting of swelling, mottling, fatty degeneration and even a necrotic appearance, along with congestion, variously sized subcapsular haemorrhages and occasional focal or diffuse mononuclear and heterophilic infiltration in the hepatic parenchyma, along with intranuclear inclusion bodies in hepatocytes (Grewal *et al.*, 1981; Reece *et al.*, 1986b; Mishra, 1988, Christensen and Saiffudin, 1989). These workers also recorded 1–14% mortality (usually less than 2%) and a course varying from 1 to 4 weeks. Mousa and colleagues (1984) and Reece and colleagues (1986a) recorded IBH cases complicated by infectious bursal disease, coccidiosis, colibacillosis, paratyphoid or a respiratory disease complex. In such cases the mortality could be up to 60%. Colibacillosis, coccidiosis, mycosis, chronic respiratory disease, mycotoxicosis and Newcastle disease were also concurrently recorded with natural cases of IBH in this department (Grewal *et al.*, 1981; Mishra, 1988; Sandhu *et al.*, 1994). Increased mortality due to IBH in association with such concurrent disease may be due to the cumulative effect of stress and immunosuppression. Both IBH and mycotoxicosis affect young chicks and are immunosuppressive, so that each might be expected to predispose the birds to the other diseases and also to exacerbate the damage. An experiment was undertaken to study the interaction of IBH virus with aflatoxin B_1 and ochratoxin A.

MATERIALS AND METHODS

Experimental chicks

Day-old broiler chicks (Punjab 1 × Punjab 2) were procured from Punjab Agricultural University (PAU) hatchery. They were screened for maternal antibodies against IBH virus by AGPT using serum (Woernle, 1966) and for vertically transmitted IBH virus in cloacal swabs using a rapid indirect dot-EIA (Oberoi *et al.*, 1993). One hundred and twenty chicks found negative for both IBH virus infection and antibodies against this virus were selected for the experiment.

Mycotoxins

Aflatoxin B_1 and ochratoxin A were procured from Sigma Chemical Co., St Louis, MO, USA. Each mycotoxin was separately dissolved in analytical-grade chloroform and individually mixed with the ration to obtain the required concentration in the feed. The feed was kept for a few days before being given to the birds to allow the chloroform to evaporate. Then the aflatoxin B_1 and ochratoxin A were extracted from a sample of feed by the methods of Romer (1975) and Hald and Krogh (1975), respectively. The extracts were analysed by thin-layer chromatography to check that

the concentration of each mycotoxin was that desired: 0.5 ppm of ochratoxin A and 1.25 ppm of aflatoxin B_1.

Inclusion body hepatitis virus

IBH virus of serotype 1 was procured from the Department of Veterinary Bacteriology and Virology, PAU, Ludhiana, India. This virus had been isolated from the livers of birds from a natural outbreak of IBH.

Experimental design

The birds were divided into 6 groups of 20 chicks each. The treatments given to different groups are detailed in Table I.

Management of experimental birds

The chicks were kept in cages with feed and water available *ad libitum*. Those in groups V, AV and OV were kept in a separate room in the same building and were routinely fed and watered after the chicks that had not been infected with the virus had been attended to.

TABLE I
Experimental design

Group	No. of chicks	Treatment
C	20	Nil
A	20	Aflatoxin B_1, 1.25 ppm, fed from 3 to 38 days of age
O	20	Ochratoxin A, 0.5 ppm, fed from 3 to 38 days of age
V	20	1.0 ml of IBH virus, of a titre of $\log_{10}6.5$ EID_{50} per 0.2 ml intra-abdominally at 10 days of age
AV	20	Both IBH virus and aflatoxin B_1 as above
OV	20	Both IBH virus and ochratoxin A as above

TABLE 2
Severity and number of birds having different type of lesions in each group at different intervals

Tissue/lesion	3 DPI/10 DPF					8 DPI/15 DPF					18 DPI/25 DPF					28 DPI/35 DPF				
	A	O	V	AV	OV	A	O	V	AV	OV	A	O	V	AV	OV	A	O	V	AV	OV
Liver																				
Granular degeneration	M/4[a]	S/3	M/4	M/3 S/1	S/4	M/3	M/3 S/1	M/4 S/1	M/2 S/2	S/2	M/4	M/2 S/2	M/4	M/1 S/3	M/1 S/2	S/3	S/3	M/3	M/3 S/2	S/2
Vacuolar degeneration	m/3	m/1	M/3 S/1	M/4	M/3 S/1	m/1 M/1	m/1	M/2	M/4	M/2	m/2 S/1	m/1	m/2	M/3	M/2	M/3	m/2	m/1	m/2 M/3	m/1 M/1
Necrosis	–	–	m/1	m/2	M/1	m/1	–	m/2 M/1	m/3 M/1	m/2 M/1	m/3 S/1	–	–	M/2 S/1	m/1	m/3 M/2	m/1	–	m/2 M/3	m/1
Inclusion bodies	–	–	m/3	M/3	m/1 M/2	–	–	m/2	M/3	m/2 M/1	–	–	–	–	–	–	–	–	–	–
Congestion and haemorrhage	m/3	m/2 M/1	M/4	m/3	m/1 M/1	m/2 M/1	m/3	m/4 M/1	m/2 M/3	m/4 M/1	m/3 M/2	m/2 M/1	m/4	m/3 M/2	m/2 M/2	M/4	m/2	m/2	M/3 S/1	m/3
Kidney																				
Granular degeneration	M/4	S/3	M/2 S/1	M/2 S/2	S/3	M/2 S/1	M/2 S/2	M/3 S/1	S/3	S/3	S/3	S/3	M/4	S/1 M/2	S/3	S/2	m/1 S/3	M/4	M/2 S/3	S/4
Vacuolar degeneration	m/2	m/1 M/3	M/2 S/1	M/3 S/1	m/3 M/2	m/3 M/1	m/3 M/1	m/2 M/2	S/3	M/1 S/3	m/2 S/1	m/2 M/2	M/4	M/2 S/1	m/1 S/2	m/2 M/4	m/1 M/3	m/2	M/3 S/2	m/3 M/2
Desquamation from basement membrane	m/2 M/1	m/2	M/2	M/2	M/3	M/3	m/1 M/2	M/1 S/1	S/3	M/2 S/2	S/1	m/2 M/2	m/4	M/1 S/1	M/3	M/2 S/1	m/1 M/2 S/1	m/2	m/2 M/3	M/2 S/2
Congestion and haemorrhage	m/3 M/1	m/2 M/2	m/4	m/3 M/2	m/3 M/1	m/2 M/1	m/3 S/1	m/3 M/2	m/2 M/3	m/2 M/3	M/2 S/1	m/3 M/2	m/4	M/4 S/1	M/3	M/3	m/3 M/2 S/1	m/4	m/1 M/3 S/1	M/3

m = mild lesion; M = moderate lesion; S = severe lesion
[a]Number of birds with lesions

The birds were closely monitored for clinical signs. Five birds from each group were killed by craniocervical dislocation at 10, 15, 25 and 35 days after first being fed mycotoxin-contaminated feed (DPF) and 3, 8, 18 and 28 days after inoculation of IBH (DPI), respectively.

Tissue collection and processing

The birds were necropsied and any gross lesions were recorded. Pieces of tissue from the liver, kidneys, spleen, lungs, heart, bursa of Fabricius, proventriculus, small intestine, thymus and brain were collected and preserved in 10% buffered formalin. They were later processed, sectioned and stained with haemotoxylin and eosin using routine techniques.

RESULTS

Gross changes

Mildly enlarged and pale livers and kidneys were observed in the IBH-infected birds up to 18 DPI. No appreciable gross changes were observed in the aflatoxin-fed and ochratoxin-fed birds except for gradual enlargement of the kidneys from 10 DPF onwards and visceral gout in a few (2/5) birds in group O. The lesions were slightly more severe in the combined groups, AV and OV, than in groups A or O (Table II). However, the severity of the gross lesions in the livers of groups AV, OV and V were comparable.

Histopathology

The numbers of affected birds and the severity of various types of lesions in each group at different times are shown in Table II.

In IBH, varying degrees of degenerative changes, congestion and mild haemorrhages were seen in the liver, together with occasional lymphoid cell infiltration and bile duct proliferation, mostly up to 18 DPI but in some cases up to 28 DPI. Large, round inclusion bodies, mostly basophilic, surrounded by a clear halo with marginated chromatin and nucleolus were discernible up to 8 DPI (Figure 1). In the kidneys, various degenerative changes of the renal tubular epithelium, slight to moderately contracted glomeruli, congestion and mild haemorrhages were consistent changes, along with regeneration of the tubular epithelium in some places. There was mild depletion of lymphocytes up to 18 DPI in the bursa. Mild reticular cell proliferation was seen in the spleen from 3 to 28 DPI. Congestion and slight haemorrhages were observed in the myocardium and lungs.

In the aflatoxin-fed birds in group A, granular followed by vascular degeneration were the most prominent microscopic changes in liver. However, necrosis of hepatocytes with marginated chromatin in their nuclei and proliferative bile duct epithelium were seen in some cases. Moderate congestion and mild to moderate haemorrhages were frequent (Figure 2). In the kidneys, granular and mild vacuolar

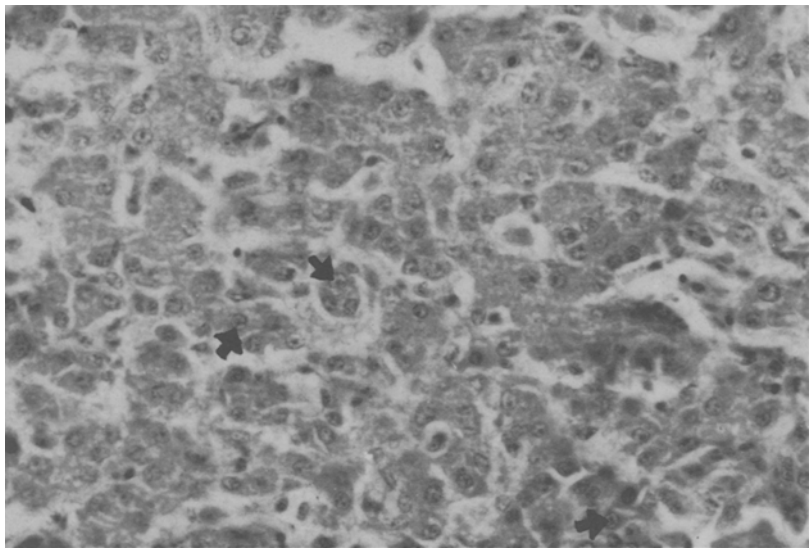

Figure 1. Group V (liver). Granular and vacuolar degeneration along with intranuclear inclusion bodies (arrows). H&E, ×400

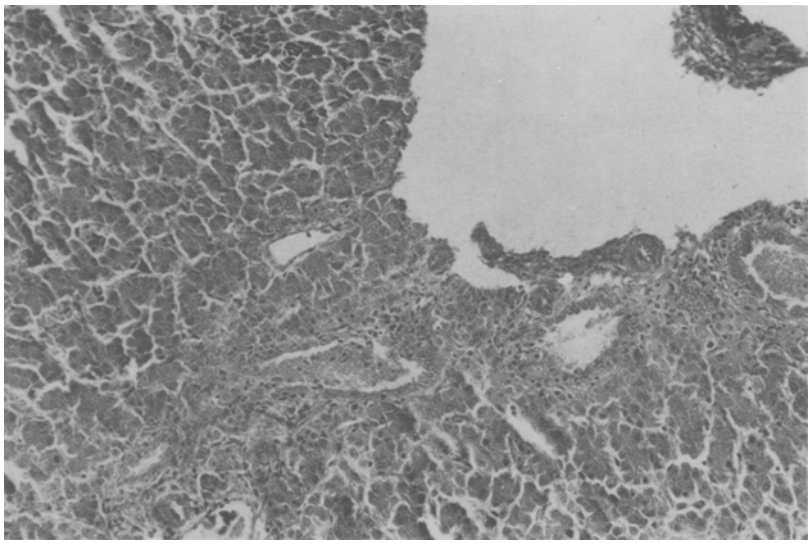

Figure 2. Group A (liver). Granular degeneration in hepatocytes along with bile duct proliferation. H&E, ×200

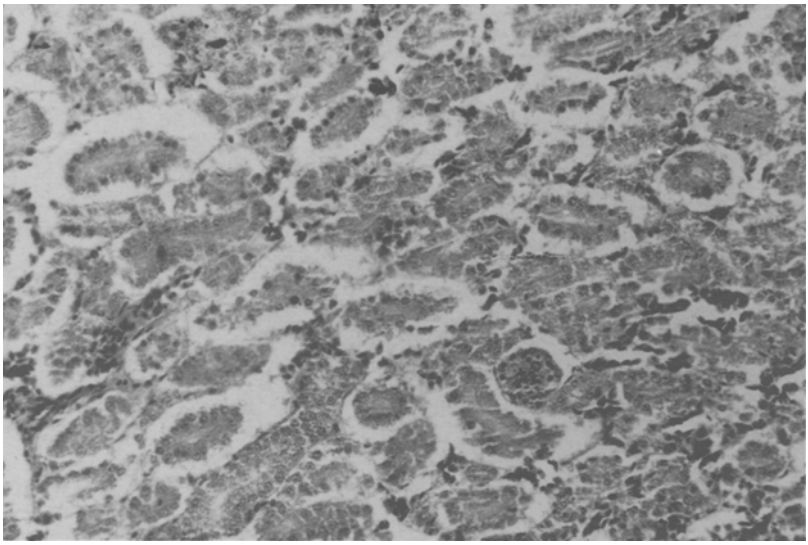

Figure 3. Group A (kidney). Extensive granular degeneration of tubular epithelium along with desquamation of epithelial cells from the basement membrane. Congestion and haemorrhages also seen. H&E, ×200

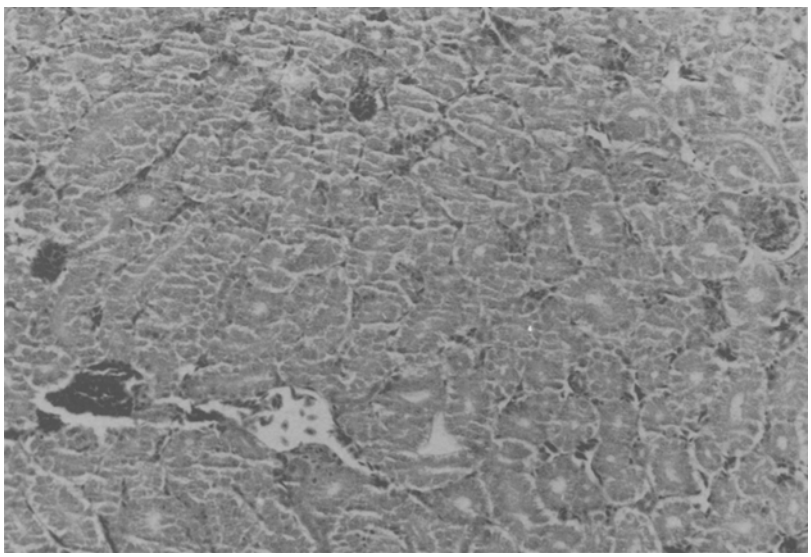

Figure 4. Group O (kidney). Extensive degeneration along with necrosis and individualization of tubular epithelial cells. Protein material precipitated into the lumina of the tubules. Congestion and haemorrhages also seen. H&E, ×150

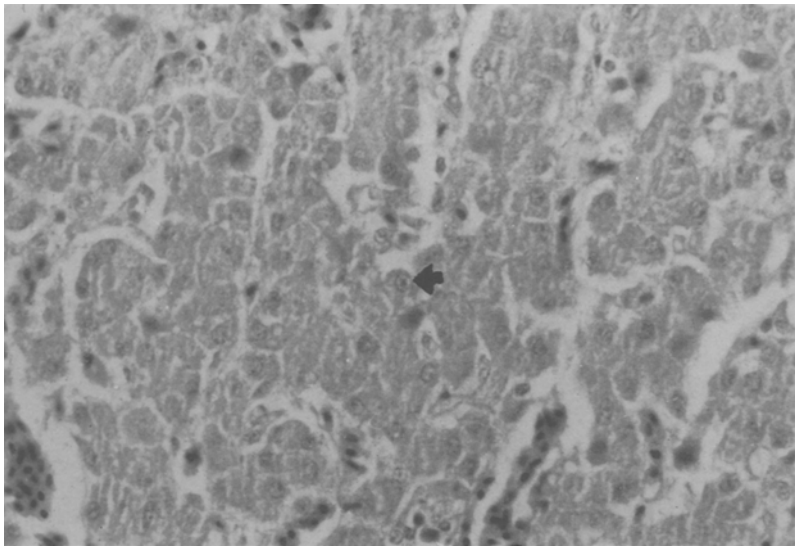

Figure 5. Group AV (liver). Extensive vacuolar degeneration and necrosis of hepatocytes with intranuclear inclusion bodies (arrow). Mild haemorrhages also seen. H&E, ×400

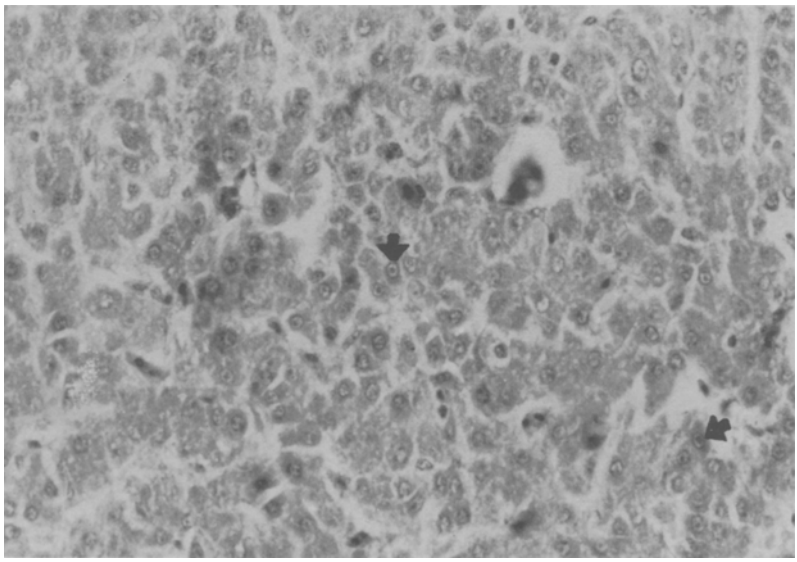

Figure 6. Group OV (liver). Granular and vacuolar degeneration of hepatocytes with intranuclear inclusion bodies. H&E, ×400

degeneration and epithelial cell desquamation in the tubules were the predominating lesions. In some of the tubules, the epithelial cells attained the appearance of columnar epithelial cells, individually projecting from the basement membrane. Mild rarefaction of the lymphoid cells in the bursal follicles and the spleen was observed in some of the birds. The damage was more severe in the later stages (Figure 3).

The liver of the ochratoxin-fed birds (group O) had varying degrees of granular degeneration along with mild congestion and haemorrhages from 10 DPF onwards. However, vacuolar degeneration and lymphoid aggregates were seen in a few cases in the later stages. Ochratoxin mainly affected the kidneys, in which congestion and haemorrhages were seen in 18 out of the 20 chicks in group O. The cellular damage gradually increased from 10 DPF onwards, with varying degrees of granular and vacuolar degeneration of the tubular epithelium, followed by desquamation leading to the formation of pseudogiant cells, mostly at 25 and 35 DPF. Contracted glomeruli and increased urate deposition in the ureters were also evident during these stages (Figure 4). Attempts at regeneration of the renal tubules were recorded with increased frequency from 10 DPF onwards. Depletion of the lymphoid cells in the bursa and spleen, along with proliferation of reticulo-endothelial cells was recorded from 15 DPF.

Microscopically, the damage in the combined group AV was comparable to that in group V but more pronounced than in the uninfected aflatoxin-fed birds (group A) up to 8 DPI/15 DPF. Subsequently, the changes in this combined group were more marked than in the individual group. Intranuclear inclusion bodies in the liver were more prominent and frequent at 3 and 8 DPI. Prominent and proliferative bile duct epithelium was recorded more consistently than in individual treatment groups (Figure 5). The degenerative changes, congestion and haemorrhages in the kidneys were also more severe in the combined group AV than in groups A or V. However, there were no appreciable differences in the severity or extent of the microscopic lesions in the other organs of the different groups, except that there was more lymphoid cell depletion and crypt formation in the bursa in a few cases at 28 DPI and 35 DPF in group AV as compared to groups A or V.

In group OV, the histopathological changes also tended to be slightly more severe than in the groups infected with IBH or fed ochratoxin A alone at 18 and 28 DPI (Figure 6).

DISCUSSION

Fadly and colleagues (1976) recorded similar gross lesions in 3 out of 10 SPF birds infected with the Tipton strain of IBH virus. However, most other workers (Helmboldt and Frazier, 1963; Hoffman *et al.*, 1975; Grewal *et al.*, 1981; Reece *et al.*, 1986a) have recorded comparatively severe gross lesions in various organs. Using comparable routes and durations of aflatoxin B_1 feeding, different workers have described no lesions or such mild gross lesions as enlarged liver, kidney, spleen and gallbladder along with occasional petechiae on the liver, kidneys and muscles (Smith and Hamilton, 1970; Chen *et al.*, 1984; Giambrone *et al.*, 1985). Pale and enlarged kidneys and liver along with haemorrhages on the liver were the gross findings by other workers during ochratoxicosis in broilers (Huff and Hamilton, 1974; Hamilton *et al.*, 1982; Dwivedi and Burns, 1984). Slight differences in the severity of lesions in

the combined treatment groups compared with their respective individual groups is indicative of a lack of any major interaction between the toxin and the virus.

Relatively severe histopathological changes have been recorded in various organs by various workers in natural and experimental cases of IBH (Helmboldt and Frazier, 1963; Hoffmann *et al.*, 1975; Grimes *et al.*, 1978; Grewal *et al.*, 1981; Reece *et al.*, 1986a; Mishra, 1988). The aflatoxin-fed group had a similar pattern of histopathological changes to that observed by Smith and Hamilton (1970); Chattopadyay and colleagues (1985); Balachandran and Ramakrishan (1987); Okoye and colleagues (1988) and Raina and Singh (1991) in various organs of chicks fed toxin at comparable dose rates up to 3–5 weeks of age. Other workers have recorded similar changes during ochratoxicosis, and also localized necrosis, fatty changes and the presence of hyaline casts in kidneys, lysis and pyknosis of ganglionic cells in the brain and changes in the myocardium and lungs at comparable dose rates (Peckham *et al.*, 1971; Huff *et al.*, 1975; Pepeljnjak *et al.*, 1982; Dwivedi and Burns, 1984; Dwivedi *et al.*, 1984; Raina and Singh, 1991). The predominant renal damage caused by ochratoxin is in accordance with its well-known property of renal toxicity.

It was inferred from this study that the effects of ingesting aflatoxin B_1 or ochratoxin A and of concurrent IBH infection were generally additive, as evidenced from the more marked histological changes in the combined treatment groups (AV and OV) than in the individual groups (A, O and V) studied in this experiment and elsewhere (Singh *et al.*, 1994). However, the severity of the histopathological lesions in these experimental groups was less than that described in field outbreaks of IBH alone or in association with mycotoxicosis (Sandhu *et al.*, 1994). This may be due to variations in the virulence of the virus or in the level of the mycotoxin in the feed, or to stress due to managemental and environmental factors in the field outbreaks that could not be duplicated in the experimental studies.

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