Journal of Veterinary and Animal Sciences. 25 (1994) 19-24

EFFECT OF DIETARY PROTEIN LEVELS ON AFLATOXIN - INDUCED HEPATOCARCINOGENESIS IN PIGS

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Aflatoxins are highly potent carcinogenic mycotoxins and comparative studies in laboratory animals have given evidence on the superior hepatocarcinogenic potential of aflatoxin B_1 among several known chemical carcinogens (Dickens and Jones 1963, Kraybill and Shimkin, 1964). Aflatoxin B_1, B_2, G_1 and their metabolites M_1 and M_4 have been demonstrated to induce tumors in hepatic and extrahepatic tissues of animals (Butler *et al.*, 1969, Lafont *et al.*, 1989). Contamination of human diet with aflatoxins have caused hepatitis and deaths in human beings (Krishnamachari, 1975). Causal association of aflatoxins with human primary hepatocellular carcinomas has been suspected and monkeys were used as experimental models for evaluating the carcinogenic potential of aflatoxin in non-human primates. Dietary modification by using low and high levels of proteins in the diet of monkeys modified the carcinogenic effects of aflatoxin B_1 (Mathur *et al.*, 1991). Pigs are very susceptible to acute and chronic forms of aflatoxicosis (Gopalakrishnan *et al.*, 1987; Rajan *et al.*, 1989). Spontaneous cases of hepatic carcinomas due to consumption of aflatoxin contaminated feed were reported in pigs (Chauhan *et al.* 1984). The effect of two levels of protein in the diet on aflatoxin induced hepatic carcinogenesis was investigated in the present study.

MATERIALS AND METHODS

Experimental animals and feed

Twenty four healthy, Large White Yorkshire male pigs of 3-4 months' age were randomly selected from the University Pig Breeding Farm, Mannuthy. The animals were divided into four equal groups and were stationed in separate sties. Two groups were treated as the test groups and other two were kept as controls.

The treatment for the four groups was as follows:

Group I	-	High protein diet and aflatoxin B_1
Group II	-	Low protein diet and aflatoxin B_1
Group III	-	High protein diet alone
Group IV	-	Low protein diet alone

The feed for the pigs were compounded locally and the ingredients were tested and ensured to be free of aflatoxin, ochratoxin and citrinin. The ingredients used to prepare the concentrate feed containing 22% protein were; yellow maize (36%), rice bran (10%) dried unsalted fish (15%), gingelly cake (38%) and mineral mixture (1%). Low protein diet was

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prepared by mixing yellow maize (44%) rice bran (15%) dried tapioca chips (20%) dried unsalted fish (15%) gingelly cade (5%) and minerala mixture (1%). Ih both cases, vitablend AB_2, D_3 (20 g) and common salt (250 g) were added to every 100 kg of feed mixture. The aflatoxin used for the experiments was prepared in the laboratory by culturing a toxigenic strain of *Asergillus parasiticus* on rice. Semipurified aflatoxin was prepared and the concentration of aflatoxin B_1 was estimated adopting the method recommended by Pons and associates (1973) with modifications, standard aflatoxins received from Makor chemicals, Israel were used to compare fluorescence on ultraviolet radiation.

Administration of aflatoxin:

The purity of the extract was estimated as 50% of AFB.1. The chloroform was evaporated and each 10 g residue was dissolved in 200 ml of 95% of ethyl alcohol. The dissolved toxin was given daily *per os* to the pigs after adding the required dose of toxin in 50 g quantity of concentrates. A quantity equivalent to 25 g/kg b. wt. aflatoxin B_1 was fed daily to the pigs in groups I and II for a period of 36 weeks. The animals were kept under observation over one year and were sacrificed for detailed autopsy examination and collection of tissue samples for histopathologic and ultra-structural studies. Neutral formalin (10%) and 3% glutaraldehyde buffered with cadodylate were used as fixatives. Paraffin sections cut at 4 μ thickness were stained with haematoxylin and eosin for histological examination of tissues.

The important clinical parameters evaluated during the period of observation were, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, blood coagulation time and haemogram profile at monthly intervals. The estimation of enzymes and bilirubin was done using commercially available kits (Miles Indian Ltd. Baroda) in Chemetrics Analyser. Standard laboratory procedures were employed for haemogram studies and blood coagulation time estimation, (Wintrobe, 1964).

Results and Discussion

In the experimental group (Gr.I) three animals survived the first year of experiment. The others died of nonspecific diseases before completing one year under observation and hepatic lesions were not seen in them. Significant increase in the serum concentration of ALT and AST occurred in the experimental animals towards the terminal period (56 weeks). Leucocytosis was observed from the 10th month onwards and it lasted till the end of the experiment. An appreciable lymphocytosis was seen after the 12th month. The liver was pale brown and fairly hard in two animals. A soft white nodule of 5 mm diameter was seen almost at the centre of the left lobe in one of the animals. Another similar nodule was observed in the parenchyma adjacent to the gall bladder. Cut surfaces of the liver showed pale streaks and patches in several areas. Similar lesions were seen in the second animal also.

No such growth was seen in the third animal. The gall bladder in all the animals were distended with yellowish green bile. The hepatic lymph nodes did not show any visible lesion. The organs and tissues of the control pigs (Gr. III) did not show any gross lesion.

In the group II animals, there was an increase in the ESR from the 6th month onwards. Leucocytosis was seen from the fourth month. There was increase in the AST and ALT from the third month. Serum bilirubin was elevated from the sixth month. Clinically the animals were apparently healthy. The livers of all the test

animals were moderately hard and pale brown. A soft greyish white nodule of 0.5 mm diameter was observed in the liver parenchyma in one of the animals. No lesions were observed in other pigs. The gall bladder was moderately distended with yellowish green bile. The gastric mucosa of the experimental pigs were moderately congested and thickened several fold than those of the controls. In three animals including the one which had the hepatic tumor, the mucosal thickening was very severe and the mucosa was thrown into folds to form corrugations. Small ulcers were present on the surface of the folds. The mucosa of the duodenum also was moderately congested and thickened. The mesenteric lymph nodes were moderately swollen and oedematous. Focal areas of pallor extending into the medulla and distinct white spots were present on the renal cortex of three test animals.

The organs of the control pigs did not present any lesion.

Histopathological examination

Focal fibrous tissue proliferation surrounding few hyperchromatic pleomorphic cells with large nuclei and prominent nucleoli were seen in the portal areas of several hepatic lobules. Some of the typical megalocytes were multinucleated. In occasional foci, groups of such hyperchromatic hepatocytes were surrounded by large number of lymphocytes and young fibroblasts. Microscopic foci of rounded disassociated hepatocytes with hyperchromatic cytoplasm and prominent nucleus and nucleoli were present in several hepatic lobules.

The nodular growths (Gr.I) were composed of large polyhedral cells with hyperchromatic cytoplasm, enlarged vesicular nucleus and distinct nucleoli, arranged as non-coherent sheets and encircled islands. Multinucleated and pleomorphic cells and mitotic figures were occasionally present (Fig.1). In some areas the neoplastic cells infiltrated the fibrous tissue stroma in trabecular fashion. The pleomorphic, hyperchromatic, neoplastic cells formed tumor emboli in arteries, veins and lymphatics at several foci (Fig. 2). Focal infiltration of large number of lymphocytes and other mononuclear cells were seen. The overall histological features presented the picture of a well differentiated hepatocellular carcinoma with the neoplastic cells arranged in imperfect sheets, trabeculae and acinar patterns. In scattered areas, necrosis of hepatocytes and fibroblastic hyperplasia were seen. The hepatic lymph nodes of the tumour bearing animal showed presence of few neoplastic hepatocytes in the medullary region.

In the kidneys a mild degree of nephrosis involving the cells of proximal convoluted tubules was present. Moderate hyperplasia of glandular epithelium was seen in the stomach. The liver sections of group II animals revealed areas of focal fibrosis and granular degeneration of hepatocytes mostly in the periportal areas. Several foci of hepatocytomegaly, karyomegaly and few giant cells were observed. Regenerative hyperplasia was evident in several locations.

The hepatic nodule presented a different picture from that observed in the Group I animals. Large number of ovoid and elliptical cells with intense basophilic cytoplasm, enlarged basophilic nucleus and prominent nucleoli were arranged as imperfect ductules, tubules and papillary projections. Giant cells with multiple nuclei and nucleoli were occasionally present. The nuclei of most of the basophilic cells were eccentrically placed. The histological features were those of cholangiocellular carcinoma (Fig. 3).

Moderate degree of hyperplasia and hypertrophy of the glandular epithelium of the

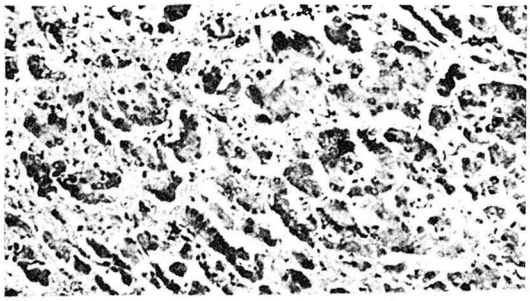

Fig.1 Hepatocellular carcinoma with multinucleated and pleomorphic cells

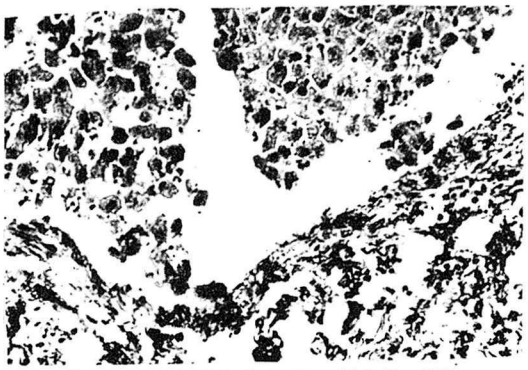

Fig.2 Tumour emboli in the artery H & E x 200

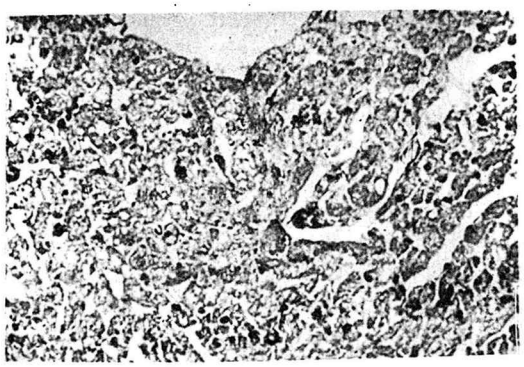

Fig.3 Cholangiocellular carcinoma H & E x 200

stomach was evident in the animals. The lining epithelium showed evidence of pressure atrophy and focal degeneration. Erosion of the epithelial lining was present in a few foci. In the intestines, goblet cell hyperplasia and mononuclear infiltration of the mucosa were seen. Focal areas of degeneration and necrosis of the epithelium of the proximal convoluted tubules and Henle's loop and focal haemorrhages were the importantchanges in the kidney. Moderate oedema and necrosis were evident in the mesenteric lymph nodes of these animals.

The observation of carcinomatous growths in two out of the three experimental animals that survived one year of experimental aflatoxin administration with a high protein diet is important. In the low protein group of animals which were given similar dose of aflatoxin, only one case of tumor development was observed. Thus it can be seen that the incidence of hepatic carcinoma was 66% in the group that received high protein diet plus aflatoxin, while it was only 16% in the low protein diet plus aflatoxin fed group. Data on haemogram, transminases, total proteins and bilirubin in serum and blood coagulation time, indicated an early and more severe onset of hepatocellular injury ie. from the fifth month onwards in the low protein group while hepatocellular alteration probably carcinomatous transformation was delayed upto one year in the high protein group.

The effect of dietary protein on aflatoxin induced liver injury was reported in weanling rats (Madhavan and Gopalan 1965). Fatty changes, bile duct proliferation and periductal fibrosis were the prominent changes in the liver of the low protein group of rats while the histologic changes in the liver of the high protein group were: swollen, vacuolated hepatocytes, pyknotic nuclei, hepatocytomegaly and hepatokaryomegaly. The nucleus of the hepatocytes was vesicular and occasionally contained prominent nucleoli. Binucleate cells were also seen. It is evident that the nature of hepatic injury in either case is different. Based on the data of similar experiments in monkeys, Madhavan *et al.* (1965) stated that dietary protein deficiency markedly enhanced the susceptibility of primates to low levels of

aflatoxin. Severe generalised fatty change was the important alteration they could observe in the liver of the low-protein group of monkeys. Such changes were not seen in the high protein group.

Mathur *et al.* (1991) observed extensive periportal necrosis of hepatocytes associated with bile duct proliferation in monkeys when aflatoxin B_1 was incorporated in a low protein diet at 1.0 ppm level. No regenerative activity could be observed in the liver. But a few foci showed cytoplasmic changes in the form of clear or acidophilic cytoplasm suggestive of preneoplastic lesions. In the high protein diet group prominent regenerating nodules showing preneoplastic alteration of hepatocytes were present. Neoplastic foci also were seen. In earlier experiments, emergence of preneoplastic lesions at 90 weeks was observed by them in the low protein group of monkeys fed 0.16 ppm of AFB_1 in diet whereas monkeys given adequate protein diet and the same dose of toxin in diet did not show any preneo-plastic/neoplastic lesions on a follow up of 120 weeks. In contrast, to the previous observations, monkeys on high protein diet and aflatoxin B_1 (1 ppm) developed neoplastic nodules in liver by 40 weeks whereas in animals on dietary restriction of proteins there were only large areas of liver cell necrosis, but no neoplastic nodules.

The experiment in pigs revealed more or less similar hepatic changes to those observed in monkeys with the high protein group of animals developing hepatic carcinomas (66%) over a period of one year even with a comparatively low level of aflatoxin intake (25 ug/kg b.wt.). The incidence of hepatic carcinoma in the low protein group with the same dosage of AFB_1 was only 16% eventhough the quantum of hepatocyte injury in the form of degeneration was more in this group. The mechanism of augmented hepatocarcinogenesis by aflatoxin with an increase in dietary protein is not established. Newberae *et al.* (1966) demonstrated that are ginine and lysine together increased the sensitivity of ducklings to aflatoxin. An enhanced levèl of enzyme synthesis by which a proximal carcinogenic metabolite is derived from the parent toxin seems possible in such situations.

The findings of the pig experiments contradict the common belief that high protein fed, well nourished animals and human beings are safe from the aflatoxin-induced liver injuries. It is evident that some amount of caution should be taken in associating aflatoxin-contaminated feed as causative factors for the incidence of primary hepatocellular carcinoma in protein deficient human population, since a high protein diet do not provide protection from hepatocarcinogenesis either in monkeys or in pigs. The presence of residual aflatoxins in the liver, kidney, blood and urine of the experimental pigs even over a month after cessation of aflatoxin administration is important (Maryamma *et al.*, 1990, 1991). The possibility of such carcinogenic toxins entering the human food chain is a matter of concern even in a population living on protein rich standard dietary regime.

SUMMARY

The influence of dietary protein levels on aflatoxin induced hepatocarcinogenesis in pigs was investigated. The pigs were given low and high levels of protein at 12 and 22% and aflatoxin B_1 was administered at the rate of 25 ug/kg b.wt. for a period of 36 weeks. Four groups of animals were maintained; group I - aflatoxin B_1 + high protein diet; group II - aflatoxin B_1 + low protein diet; group III - high protein diet alone; group IV - low protein diet alone. Each group consisted of six animals. The pigs were sacrificed after one year of the commencement of experiment. Hepatic carcinomas was recorded in 66% of the animals that survived one year on high protein diet while

incidence of hepatic carcinoma was only 16% in the low protein group. This indicated that increase in the dietary protein level did not provide protection against the carcinogenic effect of low levels of aflatoxin B_1 administered for long periods.

Acknowledgements

This study formed part of the ICAR project on Aflatoxicosis. The financial assistance provided by the ICAR is gratefully acknowledged.

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