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# Effects of Aflatoxin B1 and Poor-Protein Diet in Hamsters Infected with *Opisthorchis viverrini* *

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## อิทธิพลของ Aflatoxin $B_1$ ร่วมกับการขาดสารอาหารโปรตีนที่มีต่อหนู Hamsters ซึ่งมีพยาธิใบไม้ในตับ

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การศึกษาทำโดยนำหนูขนทอง (Golden Syrian Hamsters) จำนวน 42 ตัว มาให้อาหารโปรตีน (โปรตีน ร้อยละ 5.3) เป็นระยะเวลา 1 เดือน จึงให้ตัวอ่อนของพยาธิใบไม้ในตับ (metacercariae) 50 ตัวต่อหนูหนึ่งตัว จากนั้นป้อนสารพิษอัฟลาท็อกซิน บี. หนึ่ง ในปริมาณ 0.05 มก.ต่อสัปดาห์ติดต่อกัน 12 สัปดาห์ แล้วให้สารอาหารโปรตีนต่ำไปอีกจนสิ้นสุดการทดลอง 6 เดือน

พยาธิสภาพที่ตรวจพบได้ในตับ คือ เซลล์เยื่อบุผนังท่อนำน้ำดีเพิ่มจำนวนมากขึ้น (hyperplasia) มีการขยายตัวใหญ่ของท่อนำน้ำดีขนาดโต และมีแกรนูโลม่าเกิดขึ้น เซลล์โคลัมนาร์ของท่อนำน้ำดีเพิ่มจำนวน และซ้อนกันหลายชั้น มีการติดสีเข้มขึ้น นูเคลียสมีรูปร่างผิดปกติไป (dysplasia) ผนังเยื่อบุบางส่วนโผล่ขึ้นเข้าไปในท่อนำน้ำดีอย่างผิดปกติชนิดที่เรียกว่าพลูโดแพ็ปปิลลารีโปรเจคชั่น

เซลล์ของเนื้อตับแสดงลักษณะผิดปกติ (atypia) ถึงร้อยละ 64 ของสัตว์ทดลอง พบมีการเพิ่มขนาดของคัฟเฟอร์เซลล์เป็นหย่อม ๆ ในสัตว์ทดลองบางตัว และผลของการที่มีการเพิ่มของไฟบรัสทิชชูอย่างมากในเนื้อตับ ก่อให้เกิดโครงสร้างของตับผิดปกติไป

ระบาดวิทยาของมะเร็งตับปฐมภูมิในประเทศไทย จะได้แสดงการวิเคราะห์ โดยเฉพาะอย่างยิ่งในส่วนที่สัมพันธ์กับระบาดวิทยาของพยาธิใบไม้ในตับ ภาวะทุโภชนาและอุบัติการของสารพิษอัฟลาท็อกซินที่เจือปนอยู่ในอาหาร

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**Forty-two male Syrian Golden hamsters, maintained on a 5.3% protein diet, were experimentally infected with 50 metacercariae of Opisthorchis viverrini. An oral dose of 0.05 mg aflatoxin B1 was then administered to each animal once weekly for 12 weeks and the low protein diet further maintained for a subsequent period of 6 months.**

**Histopathological examination of the livers revealed bile ductular proliferation, dilatation of larger bile ducts with granuloma formation and pseudopapillary epithelial proliferation associated with nuclear pleomorphism, hyperchromicity and pseudostratification of the lining columnar cells. Atypia of hepatocytes was pronounced within the hepatic lobules in 64% of the hamsters. Some livers also had focal hyperplasia of Kupffer cells and parenchymal architectural alteration due to fibrous tissue proliferation within the hepatic parenchyma and around portal areas.**

**The epidemiology of primary hepatic epithelial tumours in Thailand is discussed with particular reference to the contributory roles of endemic liver fluke infestation, low-protein diet and widespread contamination of food by aflatoxin.**

## INTRODUCTION

The Statistical Report by Thai National Cancer Institute[1] indicated that on a national basis primary epithelial tumours of the liver rank highest in incidence among males (18.54% of all cancers; 3.55/100,000 population). and fourth in females (5.80% of all cancers ; 1.26/100,000 population). The hepatic cholangiocarcinoma occurs with greatest frequency in North-East Thailand whereas hepatocellular carcinoma is more prevalent in Central Thailand. It is widely believed that this differential incidence is directly related to the locally prevalent environmental carcinogens and a diversity of naturally occurring substances have been considered from this angle, e.g. hepatitis B virus, liver fluke *(Opisthorchis viverrini)*, aflatoxin, nitrosoamine, alkaloids, plant products, etc.[2-5] On the basis of clinico-pathological observations and epidemiological studies, there is strong evidence that the presence of the liver fluke is directly related to the development of intrahepatic bile duct carcinoma.[6-8]

Liver fluke infection cause by the fluke Opisthorchis viverrini is encountered in epidemic proportions among the people of North-East Thailand.[9-13] Harinasuta et al (1957) in reporting on surveys of the incidence of such infection in villagers residing in 15 provinces of North-East Thailand, showed that the average incidence of Opisthorchiasis was 61.5% (highest : 92% and lowest : 10.1%) ; 11 of the 15 provinces had an infection rate in excess of 50%. Eggs of these worms were identified in faecal specimens as early as the first year of life.

Several epidemiological studies have suggested a close geographical relationship between food contaminated with aflatoxin and the peak incidence areas of hepatocellular carcinoma in Thailand.[14-17] Aflatoxin is a potent hepatoxin and a well established hepatic carcinogen that requires metabolic activation to exert its cytotoxic and genetic effect.[18-19]

It is essential that these factors are considered against the background of the general state of nutrition of the population under study.[20] A low protein diet because of its hepatic effects might alter the pattern and the incidence of the different primary cancers in the liver by modulation of carcinogenic influence. A poor dietary protein concentration may produce a depletion in cytoplasmic enzymes, particularly those involved in activation or detoxification of many carcinogenic hydrocarbons.[22-24] The acute hepatotoxicity of aflatoxin is also enhanced with developed of a cirrhosis.[22]

As a means of investigation of the potential additive effects of a low protein diet, helminthic liver infestation and oral aflatoxin administration, the hamster was chosen as the experimental animal. This species is very susceptible to infection by worms[25-26] and

the effects of dietary manipulation were well documented in them.[23,27] Furthermore, the pathological changes in the livers of hamsters infected with Opisthorchiasis, maintained on high and low protein diet, have already been reported.

## MATERIAL AND METHOD

**Hamsters :** 42 male golden Syrian hamsters, on average 1-2 months of age and weighing about 80-100 gm at commencement of the experiment, were obtained from Animal House of Chulalongkorn University, Bangkok, Thailand.

Animals were kept 3 to a cage, and fed water and a standard pellet diet (Gold Coin Mills, Singapore : containing 25.6% protein) ad libitum.

**Diet :** At the beginning of the experiment the diet was changed to a low protein diet which contained about 5.3% crude protein. The actual formulation of this diet by percentage composition was as follows : rice flour 68%, potato starch 22%, corn starch 8%, vitamin mixture 1%, mineral mixture 1%.

All hamsters were fed this diet for the two weeks prior to receiving metacercariae and thereafter for the duration of the experiment, i.e. 6 months.

**Metacercariae and experimental infection**

Metacercariae of *Opisthorchis viverrini* were obtained from naturally infected cyprinoid fishes taken from a small village in Khon Kaen province of North-east Thailand. The fish flesh was digested by an acid/peptic technique (Pepsin A) to enable the extraction of the metacercariae ; the latter were then carefully identified and active metacercariae were selected under the dissecting microscope.

All hamsters were infected with 50 metacercariae in 1-2 ml. saline by intragastric innoculation.

**Aflatoxin B1**

Pure crystallised aflatoxin B1 obtained from Sigma Laboratory (Israel) was dissolved and diluted in dimethyl sulphoxide (DMSO) to a concentration of 0.05 mg/ml. 1 ml of above mentioned solution was dropped on to a 15-20 gm piece of prepared diet in clean Petri dish. Every week each individual animal was isolated and starved for 1-2 hours and were then presented with this toxinmixed diet and great care was being taken to ensure that all the diet was consumed by the animal.

By the end of the twelfth week every animal had received a total dose of about 0.6 mg. aflatoxin B1. The animals were maintained on a low protein diet regime.

Six months later all animals were sacrificed. The liver was removed and blocks taken from the right, middle and left lobes, were fixed in buffered formalin and stained with Haematoxylin and Eosin.

## PATHOLOGY

**Macroscopical Findings :**

The livers were smaller than normal and their outer aspect appeared shrivelled, irregular and nodulated with scar tissue in between these nodules (Fig 2). The cut surface was of a pale, reddishbrown appearance and the external nodularity corresponded to an irregular reticulated scarring of the parenchyma. Worms were identified in all lobes.

**Microscopical Findings :**

Within the larger biliary ducts both adult sexually mature flukes as well as ova were present in all the animals. Their presence was invariably accompanied by an inflammatory response of varying severity. A diffuse chronic non-specific inflammatory infiltrate composed of lymphocytes, plasma cells with occasional eosinophils, neutrophils and macrophages was invariably present within the portal triads (Fig 3). An accompanying granulomatous response was often seen in these areas the

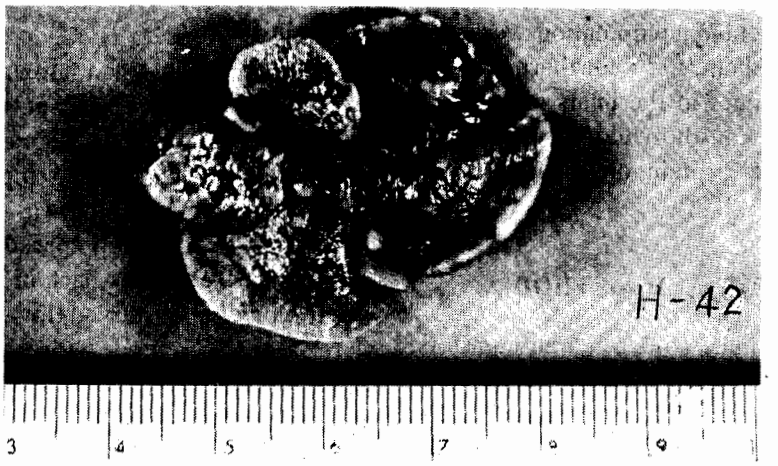
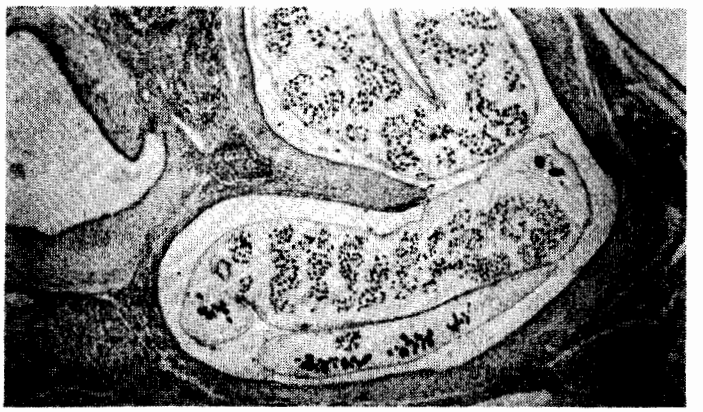
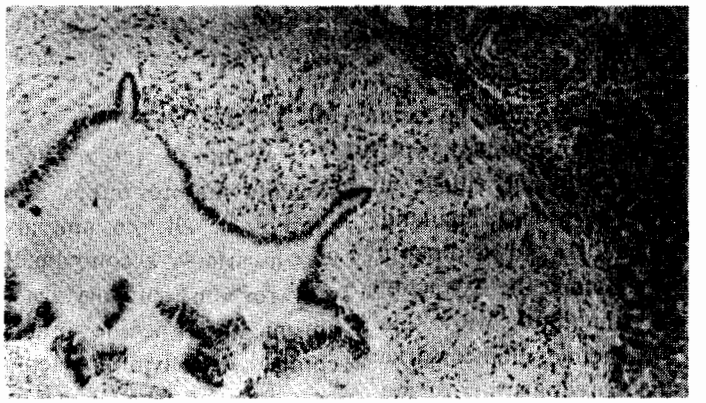



**Fig 1 Gross appearance of the hamster liver show nodulation of all the lobes with shrinkage of the hepatic parenchyma.**



**H & E × 100 HAMSTER LIVER**

**Fig 2 Adult Opisthorchis fluke within bile duct.**

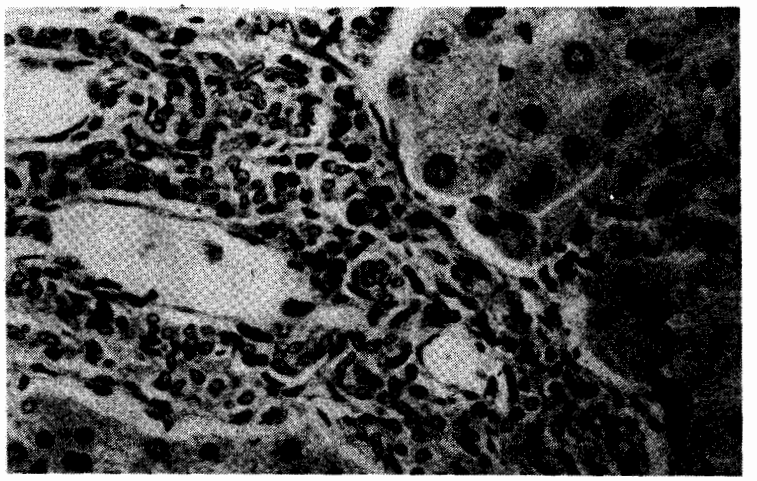


**H & E × 250 HAMSTER LIVER**

**Fig 3 Concentric fibrosis of larger bile duct with some area of granulomatous changes in a nearby bile ductule.**

macrophages were prominent and showed an epithelioid appearance with a tendency to a concentric arrangement surrounded by a halo of plasma cells and lymphocytes. No central necrosis was seen but foreign body type giant cells were prominent in a number of these lesions. Small granulomas with a less florid and more sarcoid-like appearance were also present : worm ova were present within some of these. In some areas the granulomatous lesions replaced the mucosal lining of the bile ducts. In areas of healing ulceration of the biliary epithelium, granulation tissue replaced the lining. The adult fluke in direct relationship to granulomas showed degenerative or frank necrotic changes.

The mucin secreting cuboidal or columnar epithelial cells of the biliary channels showed hyperchromasia and enlargement of these nuclei with some tendency to stratification and an increased mitotic rate : these changes were interpreted as representing an active healing and regenerative response. In other ducts the nuclei were more irregular and the cells more pleomorphic, and there was a tendency for the epithelium to be thrown out into papillary folds with a core of flimsy collagenous tissue occupying these central areas. No evidence of in-situ or invasive carcinomatous changes was present but focally the degree of dysplasia was of moderate severity (Fig 4).

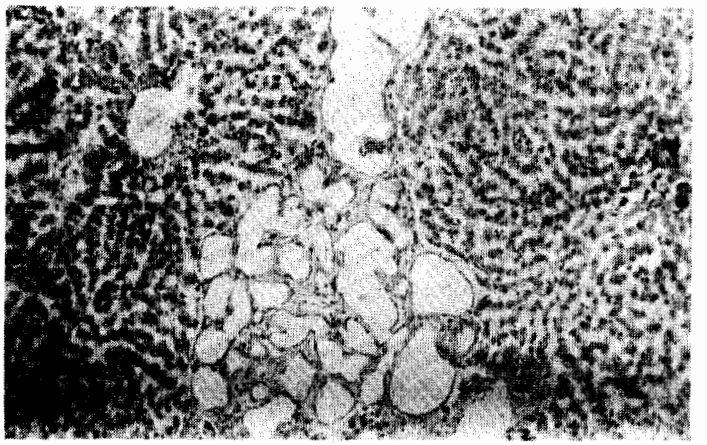


**H & E × 250 HAMSTER LIVER**

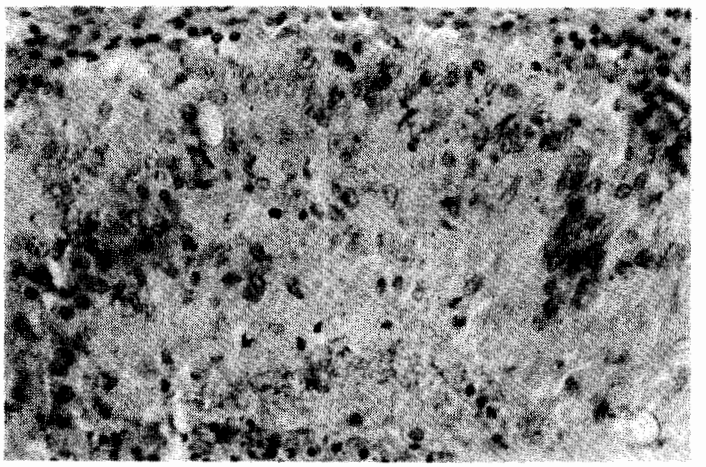
**Fig 4 Portal inflammatory changes with a mixture of acute and chronic inflammatory cells surrounding biliary and vascular channels.**

The smaller bile ducts within the portal triads appeared dilated and their epithelial lining was flattened out. A laciform or microcystic pattern was often observed (Fig 5). Similar dilatation was in the biliary canaliculi. Similarly dilated channels were particularly prominent beneath the liver capsule.

Bile stasis accompanied these changes. Bile appeared inspissated within the larger ducts and formed dense thrombi within canaliculi. The Kupffer cells were hyperplastic and often contained excessive bile pigment. Macrophages with foamy cytoplasm were also abundant within the portal triads and similar pigment was present within their cytoplasm. The degree of intrahepatic cholestasis varied with different animals and individual hepatic lobes.



**H & E × 250 HAMSTER LIVER**
**Fig 5 Laciform arrangement of dilated smaller bile ducts within portal triad.**



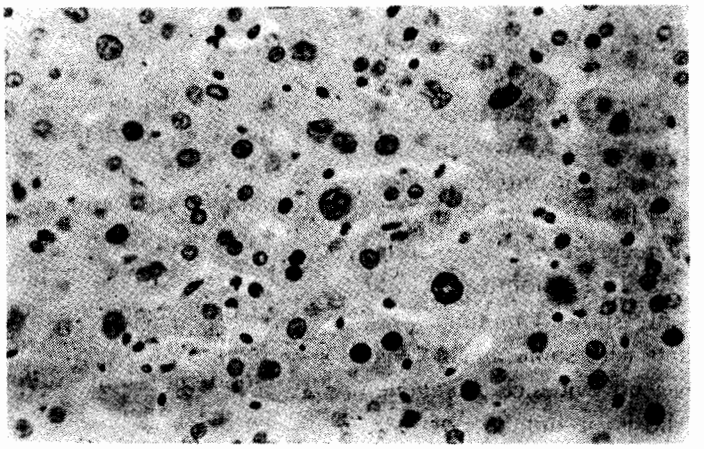
**H & E × 400 HAMSTER LIVER**
**Fig 6 Biliary epithelium showing pleomorphic and irregular of nuclei as dysplasia of moderate severity.**

A portal triaditis was present in 90% of the animals. There was, however, spill-over of inflammatory cells into the hepatic lobules and no piecemeal necrosis was seen.

The larger bile ducts showed around them an excessive concentric fibrosis composed of mature fibro-collagenous tissue (Fig 6). The portal triads also showed excessive fibrosis in 66% of the animals and from their angles there were extensions of fibrous connective tissue septa through the limiting plate. As a result small islands of hepatocytes appeared to have become dissociated from the periphery of the lobules. In two-thirds of the animals there was prominent connective tissue bridging between portal triads producing a geometrical pattern in the liver parenchyma. Central hyaline sclerosis was observed in one animal only. No

other vascular abnormalities were seen. No evidence of cirrhosis was seen in any of the animals.

Within 27 of the animals the liver lobules showed pronounced atypia of the hepatocytes (Fig 7) : the cells were often binucleated. The nuclei were larger, rounded and vesicular but the nuclear/cytoplasmic ratio was only marginally increased. They contained several very prominent eosinophilic nucleoli with an irregular outline. The nuclear membranes were irregular and the chromatin stippled. There was no particular distribution pattern of these cells and they were found with similar frequency in the periportal centrilobular and midzonal areas.



**H & E × 400 HAMSTER LIVER**
**Fig 7 Liver cells showing dysplastic changes with enlargement and hyperchromicity, some binucleate of nuclei and prominence and irregularity of nucleoli.**

In more than half of the animals the cytoplasm of the liver cells showed degenerative changes, mostly a hydropic change. No fatty infiltration was seen.

Very few, isolated and tiny foci of necrosis were irregularly distributed throughout the lobules and surrounded by a few neutrophils. These cells were structureless and their nuclei pyknotic.

No eggs, flukes or granulomas were identified within the hepatic lobules.

## DISCUSSION

The choice of the hamster as the experimental animal model was guided by the well-established susceptibility of this species to infection with worms[25-26] and the previously reported hepatic changes of these animals following maintenance on a low protein diet.[23-27] The presence of flukes in the biliary passages is known to result in proliferation and desquamation of the biliary epithelium, concentric fibrous thickening of the bile ducts, progressive ductal dilatation with eventual cyst formation and granulomatous changes.[2,9,28] The actual pathogenetic mechanisms of these changes are unknown but probably represent a combination of local mechanical irritation produced by the physical presence of the fluke, the excretion of chemical product locally by the worms and the complex interaction between the parasite and the immune system of the host.[29] Hamsters fed on a low protein diet were shown to develop liver cell atrophy, focal hepatocytic necrosis with the accumulation of lipofuscin and hae-

mosiderin. The presence of such a dietary deficiency accentuated the degree of bile ductular proliferation induced by O. viverrini infection.

Aflatoxin is a well-recognised hepatic carcinogen[3,18,19] which is converted by microsomal mixed-function oxidase concentrated in hepatocytes to aflatoxin -2 -3 oxide ; this can bind to DNA and exert a carcinogenetic effect[30,31] whose potential varies with different animal species. Hamsters are particularly resistant to its carcinogenic effect[32-33], the oral LD50 was estimated by Wogan[34] as 10.2 mg/Kg body weight, and intraperitoneal dose as 6 mg/Kg by Elis. Herrold[35] showed that hamsters which received 0.1 mg of aflatoxin twice weekly for 10-11 months, (a total dose of 8-10 mg) only showed minimal proliferation of bile ducts with some megaloblastosis of hepatocytes ; no evidence of regeneration nodules, cirrhosis or tumours of liver were observed. Moore et al[36] could induce cholangiocarcinoma in hamsters in significant proportions when a very large dose of aflatoxin (daily dose of 2 mg/Kg body weight, 5 days/ week for 6 weeks) was administered continuously for as long as 78 weeks.

Madhavan and Gopalan[37] have speculated that the loss of lipotrope from the liver cells as a result of a depleted protein diet may decrease the metabolism of aflatoxin and hence its potential carcinogenic effect. In our experiment these findings are not confirmed in that a much smaller dose of aflatoxin (0.05 mg/week for 12 weeks with a total dose of 0.6 mg) was fed to the animals and yet pronounced atypia of hepatocytes and less marked biliary dysplasia were identified. This suggests an enhanced noxious effect on the liver cells by aflatoxin in the presence of the fluke infection.

No biliary or hepatocytic tumours were identified. However, premalignant changes were observed in both cell types. It is perhaps speculative to suggest that given a further prolongation of the experiment actual tumours would have been induced.

The association of dietary deficiencies, worm infestation and exposure to aflatoxins is prevalent in many areas of the world with a high incidence of primary hepatic carcinoma.[7,38,39] In Thailand the epidemiological distribution of liver cancer is particularly interesting in that cholangio-carcinoma is common in the Northeast whereas primary hepato-cellular carcinoma is frequent in the central part of the country.[40] The Northeast has the highest density population and of protein-caloric malnutrition.[41] Liver fluke infestation is also endemic in this part of the country and an estimated 7 to 8 million people suffer from Opisthorchiasis. Shank et al carried out an investigation of the degree of contamination by aflatoxin of various foods and food-stuffs in different geographical regions of Thailand. They showed that peanuts were the most frequent source of contamination with the highest aflatoxin concentration in those samples obtained from the North eastern areas : 63% of peanuts were contaminated with a mean concentration of 735 ug aflatoxin/Kg nuts.

It is suggested that the combination of chronic biliary irritation and granulomatous inflammation produced by the worms in association with a low protein diet and aflatoxin contamination of food stuffs provides the requisite recipe for the development of primary cholangiocarcinoma. Whereas in the absence of fluke infestation a high aflatoxin concentration and a low protein diet result in hepatocellular tumours.

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