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Effect of *Clonorchis sinensis* Infection on the Histopathology of the Liver in Rats Administered Aflatoxin B_1

HYO KEEL PARK

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Abstract

The effect of aflatoxin B_1 on the sequential morphologic changes of hepatic cells was examined in the rats experimentally infected with *Clonorchis sinensis*. The Wistar strain rats were divided into three groups; group I, treated with 1.0 ppm aflatoxin B_1 alone for 12 weeks, group II, given 50 metacercariae of *Clonorchis* along and group III, given 50 metacercariae at the beginning of the treatment with 1.0 ppm aflatoxin B_1 for 12 weeks. In light and electron microscopic examination, nuclear enlargement and hyperchromasia, and nucleolar enlargement were noted in all groups. In group II cell cord disarray and focal necrosis were observed with iflammatory cell infiltration. In group III liver cirrhosis and well differentiated hepatocellular carcinoma were observed at the 28th week. These data indicated aflatoxin B_1 to possess a synergistic effect in terms of induction of neoplastic lesions in the liver infected with *Clonorchis sinensis*.

Key words: Hepatic cell carcinoma, Aflatoxin B_1, *Clonorchis sinensis*, Carcinogenesis.

Introduction

The high incidence of primary carcinoma of the liver in the Far East has been ascribed to chronic diseases caused by aflatoxins, alcohol ingestion and hepatitis B virus (Schwartz, 1980). Some fractions of aflatoxins including aflatoxin B_1 have been detected in some preserved foodstuffs and food commodities (Kim *et al.*, 1977). Aflatoxin B_1 has also been known to be one of the potent hepatotoxic and hepatocarcinogenic agents (Osuna *et al.*, 1977). Besides, the development of liver malignancies have also been attributed to parasitic infections particularly by *Clonorchis sinensis, Opisthorchis viverrini, Fasciola hepatica, Schistosoma japonicum* and *Schistosoma mansoni* (Purtilo, 1976; Bhamarapravati, 1978; Brand, 1979; Min, 1986). However, the parasite may not provide the sole carcinogenic stimulus leading to the malignancy and other exogenous carcinogenic factors, such as dimethylnitroosamine, or aflatoxin B_1 may act synergistically in the induction of carcinoma (Flavell, 1981; Min, 1986; Iida, 1985).

This study is designed to experimentally examine the sequential morphologic changes of hepatic cells in the rats given both *C. sinensis* and aflatoxin B_1 by light and electron microscopic techniques.

Department of Parasitology, College of Medicine, Ewha Womens University, Seoul, Korea

Address correspondence and reprint requests to Dr. Hyo Keel Park, #278-49 Hong Jae 3 Dong, Seo Dae Mun-Gu, Seoul, 120-093, Korea

Materials and Methods

Preparation of Metacercariae

Clonorchis sinensis metacercariae were obtained by digestion method using artificial gastric juice from freshwater fish caught from the Nagdong River in Korea. *Pseudorasbora parva* is one of the important second intermediate hosts of *C. sinensis*.

Carcinogen and Diet

The pure aflatoxin B_1 was supplied by Sigma Chemical Co., St. Louis, U.S.A. A synthetic standard protein diet without aflatoxin B_1 and the experimental diet containing aflatoxin B_1, one part per million (1.0 ppm) were prepared and supplied by the Laboratory of Sam-lip Food Co.,

Table 1 The Composition of the Diet (per kg.)

Ingredient	Quantity
Casein	180 gm
Corn oil	50 gm
Glucose monohydrate	710 gm
Salt mixture	40 gm
Riboflavin	1 gm
Vitamin mixture	5 ml*

* Contains cod liver oil 20 gm (choline chloride 1.5 gm, pteroryl glutamic acid 0.6 mg, biotin 1.5 mg, thiamine-HCl 20 mg, pyridoxine-HCl 20 mg, and menadione 50 mg in 20 gm of cod liver oil), nicotinamide 50 mg, potassium-p-aminobenzoic acid 50 mg, calcium panthothenate 60 mg, inositol 100 mg and cyanocobalamine 40 μg.

Seoul, Korea (Table 1).

Animal and Experimental Design

A total of 75 male albino rats of the Wistar strain, 8 to 10 weeks old, weighing approximately 150 gm each were used and were divided into three groups; group I, fed the experimental diet alone, group II, given 50 *Clonorchis* metacercariae alone, and group III, given 50 metacercariae at the beginning of the experiment of the treatment with the experimental diet. Additional three rats were served as non-infected and non-medicated control animals. Metacercariae were given to the animals via intragastric tube. Rats of control group and group II were fed by the normal diet. For the animsls of groups I and III the medicated experimental diet was provided *ad libitum* for 12 weeks, and then substituted by the normal diet.

Histopathological Examination

Three rats from each group were sacrificed under general ether anesthesia at 4 weeks interval up to the 28th weeks.

For the light microscopical examination the liver tissues were fixed in 10% buffered formalin solution. The representative areas were taken and embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin (H-E stain). Masson trichrome and reticulin stainings were also employed.

For the electron microscopical examination several slices of the liver were removed and minced into cubes measuring 1 mm^3 or less and fixed in phosphate buffered 1% paraformaldehyde + 2% glutaraldehyde. The tissues were washed with 4.5% sucrose solution and refixed in phosphate buffered 1% osmium tetroxide. These tissues were dehydrated in a grade series of alcohols and embedded in Epon 812. Ultrathin sections were made and mounted on titanium grids, stained with uranyl acetate and lead citrate, and examined with Hitachi H-600 electron microscope. The microscopical findings were statistically compared using X^2.

Results

At the 4 weeks liver cells of the rats in group I treated with 1.0 ppm aflatoxin B_1 showed mild nuclear enlargement and hyperchromasis (Fig. 1). These findings persisted by the 24th week, then advanced to moderate at the 28th week.

In the group Ii given 50 metacercariae a mild and moderate nuclear enlargement was observed from the 4 weeks until the 16 weeks. But nuclear hyperchromasia and nucleolar enlargement were not so distinct (Fig. 2). Focal necrosis and inflammatory cell infiltration were noted but not so prominent. A mild hepatic cell cords disarray was also revealed after the 12 week. However, no pseudolobule formation and cirrhotic changes were noted at any stage.

On the other hand, in group III treated with both *Clonorchis* metacercariae and aflatoxin B_1 much more advanced and notable changes were revealed. Hepatic cell cords disarray and inflammatory cell infiltration were moderate in the degrees at later stage. From the 4th week to the 20th week liver cells generally showed a moderate nuclear enlargement and hyperchromasia as dysplastic changes of liver cells (Fig. 3). As the other conspicuous findings pseudolobule formation and cirrhotic changes were observed in this group. In addition, well differentiated hepatocellular carcinomas, composed of hyperchromatic and dentritic hepatocytes were evidenced in two rats at the 28th week (Fig. 4).

Table 2 Summary of Light Microscopic Findings in the Liver of Rats by Group, Based on 3 Rats in Each Group at 4 week Interval

Group	Week	Liver cell						Lobule									Focal necrosis			Inflammatory cell infiltration*			Cirrhosis*		
		Enlargement*			Hyperchromasia*			Nuclear enlargement*			Cord disarray*			Pseudolobule*											
		+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
	4	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	12	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I	16	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	3	0	0	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	24	3	0	0	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	28	0	3	0	0	3	0	0	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	8	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	12	3	0	0	3	0	0	0	0	0	3	0	0	0	0	0	3	0	0	3	0	0	0	0	0
II	16	0	3	0	3	0	0	3	0	0	3	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	20	0	3	0	3	0	0	3	0	0	3	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	24	0	3	0	0	3	0	0	0	0	3	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	28	0	3	0	0	3	0	0	3	0	3	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	4	0	3	0	0	3	0	3	0	0	0	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	8	0	3	0	0	3	0	3	0	0	0	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	12	0	3	0	0	3	0	3	0	0	0	0	0	0	0	0	3	0	0	3	0	0	0	0	0
III	16	0	3	0	0	3	0	3	0	0	3	0	0	0	0	0	3	0	0	3	0	0	0	0	0
	20	0	3	0	0	3	0	3	0	0	3	0	0	3	0	0	3	0	0	3	0	0	0	0	0
	24	0	0	3	0	0	3	0	3	0	0	3	0	0	3	0	3	0	0	0	3	0	3	0	0
	28	0	0	3	0	0	3	0	3	0	0	3	0	0	3	0	3	0	0	0	3	0	0	3	0

I : Rats treated with 1.0ppm aflatoxin B_1 alone for 12 weeks.
II : Rats given 50 *Clonorchis* metacercariae alone.
III : Rats treated with 1.0 ppm aflatoxin B_1 and 50 *Clonorchis* metacercariae.
+: Mild ++: Moderate +++: Severe
The results of chin square was tested in contigency table. * < 0.01

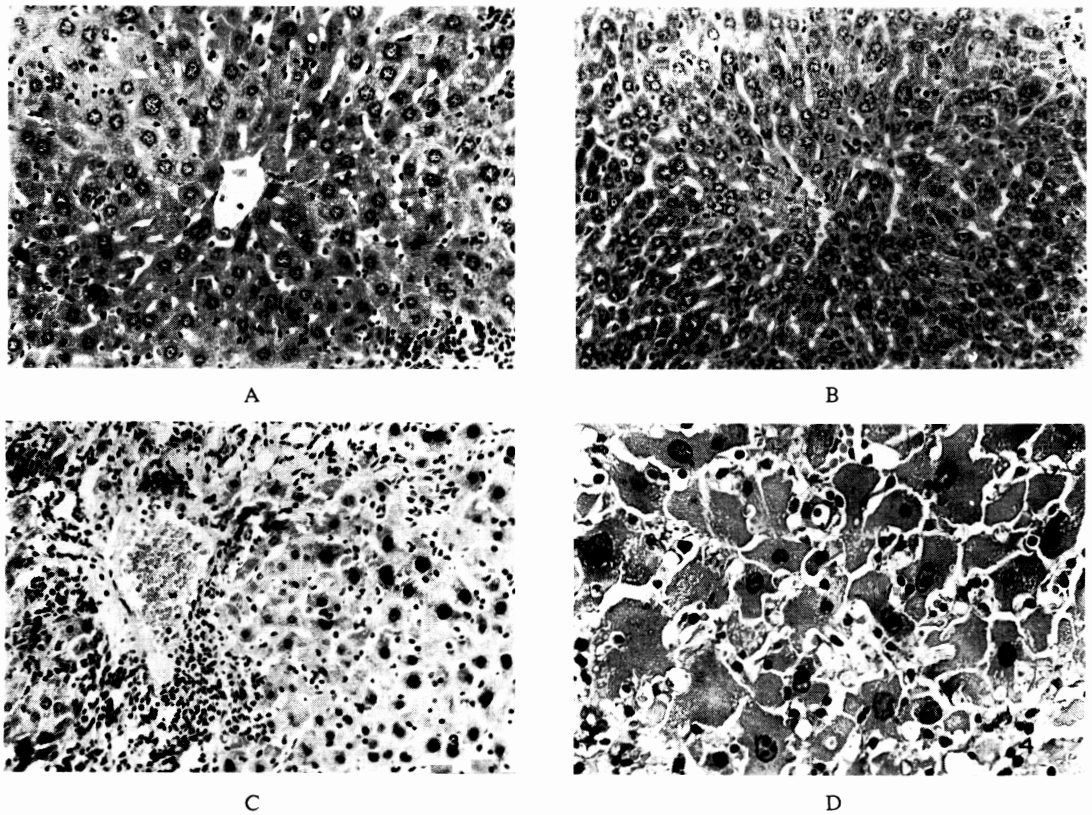

Fig. 1. A Light microscopic findings of liver cell in the rat. Treated with aflatoxin B_1 alone, at the 4th week the hepatic cell cords and lobules are intact. Mildly enlarged nuclei are seen (H-E stain, ×200).

B Given *Clonorchis* metacercariae alone, at the 16th week moderately enlarged vesicular nuclei are noted (H-E stain, ×200).

C Treated with *Clonorchis* metacercariae plus aflatoxin B_1, at the 24th week inflammatory cell infiltration, pseudolobules and cirrhosis are seen (H-E stain, ×100).

D Treated with *Clonorchis* metacercariae plus aflatoxin B_1, at the 28th week well differentiated hepatocellular carcinoma are demonstrated (H-E, ×400).

Electron microscopically, in the rats of group I, at the 16th week a mild nuclear enlargement and nucleolar prominence persisted and associated with various sized and shaped clumps of nuclear chromatin along the inner nuclear membrane. Double nuclei and multiple nucleoli were infrequently found. Rough endoplasmic reticulum and mitochondriae in cytoplasm were slightly increased in the number. Electron dense glycogen particles forming minor or major aggregates were infrequently visible. At the 28th week, nuclear enlargement, irregularity of nuclear membrane contour and chromatin condensations were frequently revealed. Hepatocytes demonstrated some enlarged nucleoli along the inner membrane of nuclear envelope, so-called nucleolar margination. Dilatation of rough endoplasmic reticulum and swelling of mitochondriae were noted (Fig. 5).

In the rats of group II, from the 16th week in a majority of hepatocytes nuclei showed moderate enlargement and cytoplasm revealed a mild dilatation of rough endoplasmic reticulum and single membrane-bound vacuoles containing granuloreticular materials. Cytoplasmic organelles such as rough endoplasmic reticulum, mitochondriae and glycogen particles showed a tendency of regression, but moderate irregularity

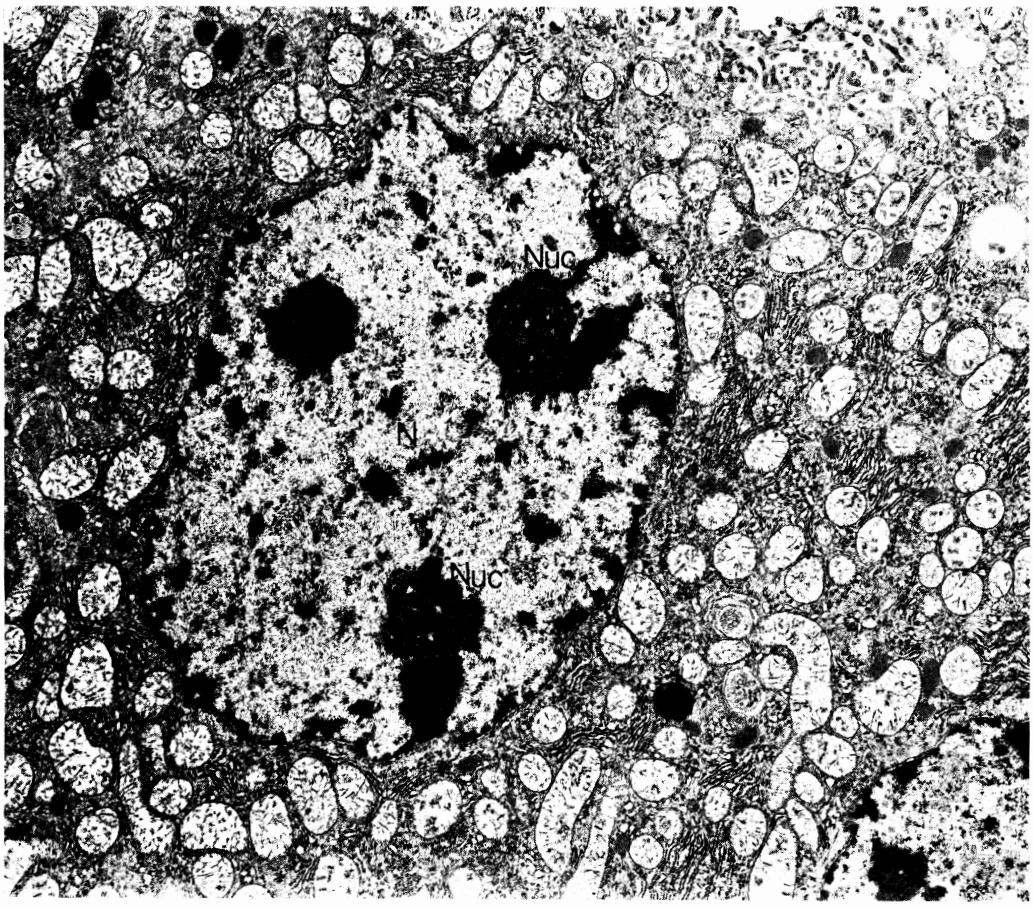

Fig. 2. Ultrastructural findings of liver cell in the rat treated with aflatoxin B_1 alone, at the 28th week three nucleoli are seen in a nucleus and two of them shifted to the inner membrane of nuclear envelope (nucleolar margination) (Lead stain, ×8,000).

of nuclear membrane contour was showed at the later stage (Fig. 6).

In the rats of group III, at the 16th week, the irregularity of nuclear membrane contour and clumps of nuclear chromatin were demonstrated. Double or multiple nuclei and nucleoli were more frequently seen and the irregularities were prominent. Those nucleoli usually demonstrated considerable modifications in conformation and structure such as fragmentation. Moderate proliferation and dilatation of rough endoplasmic reticulum irregular size and distribution of gap junctional components, and swelling of mitochondriae were seen and many electron dense granules were visible. At the 28th week, the enlargement of nuclei, clumps of nuclear chromatin and nucleolar prominence were marked. Another characteristic finding was the increased free ribosomes and the separation of the outer nuclear membrane which continued to abundant rough endoplasmic reticulum (Fig. 7). Bile canaliculi and vascular poles of hepatocytes showed the decreases of microvilli in the size and number in some parts.

Discussion

It has been strongly suggested that clonorchiasis is a causative agent in the induction of cholangiocarcinoma rather than a coincidental

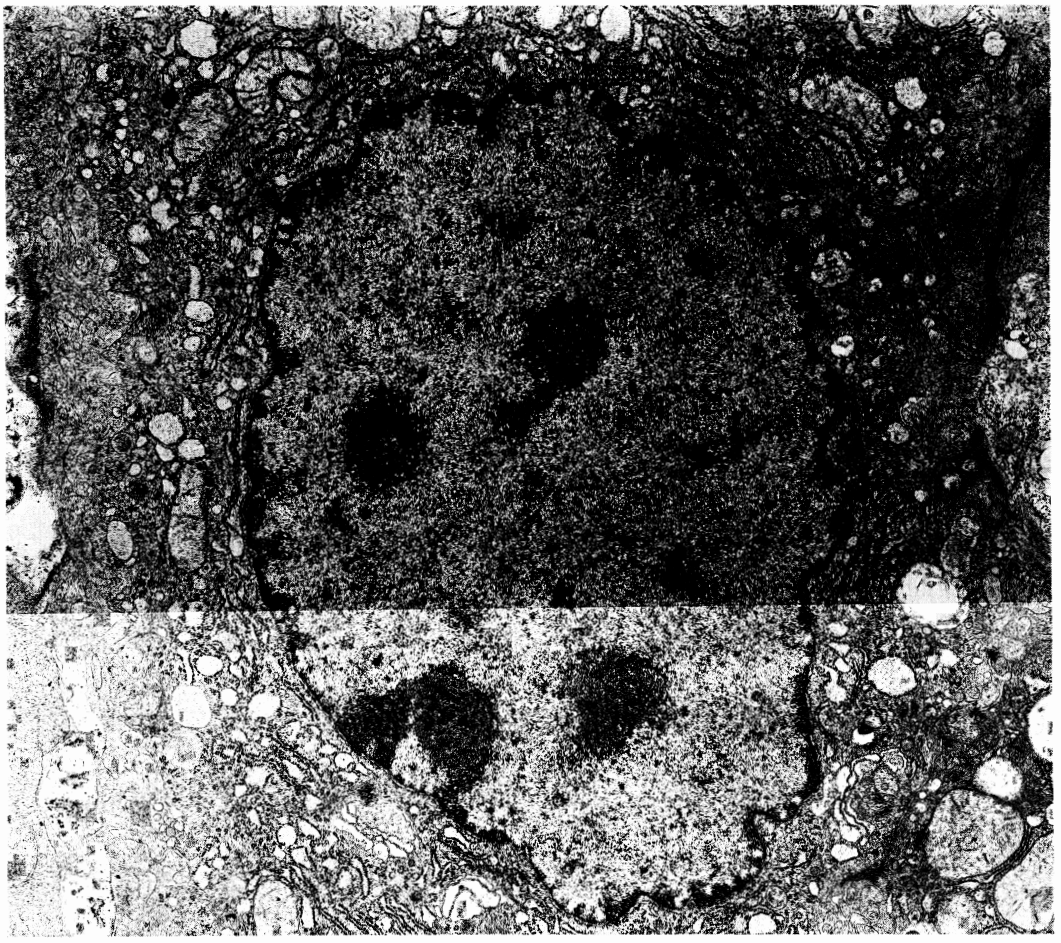

Fig. 3. Ultrastructural findings of liver cell in the rat treated with *Clonorchis* metacercariae alone, at the 28th week multiple nucleoli are seen in a moderately enlarged nucleus showing irregular membrane contour. Cytoplasmic organelles are more or less regressed (Lead stain, ×10,000).

and/or concommitant phenomenon because of the high prevalence rate of clonorchiasis and cholangiocarcinoma of the liver (Purtilo, 1976; Lee *et al.*, 1978). However, many epidemiological and pathological studies indicated that a certain exogenous carcinogenic factor may act synergistically with the liver clonorchiasis to develop cholangiocarcinoma.

Aflatoxin B_1 has been known to be an extremely potent hepatotoxic and hepatocarcinogenic agent. It is one of the metabolites of certain strains of *Aspergillus flavus* and a common mould which has been isolated world-widely from many foodstuffs (Kraybill & Shimkin, 1964; Kim *et al.*, 1977). The potential significance of aflatoxin B_1 in human liver cancer was suggested by Kraybill and Shimkin (1964).

In the present study, the rats of group II given 50 *Clonorchis* metacercariae alone demonstrated much more prominent changes in nuclei and nucleoli together with some architectural alterations of the liver, such as cord disarray and focal necrosis differently from those observed in the rats of group I treated with 1.0 ppm aflatoxin B_1 alone. Focal necrosis, particularly, produced focal nodularity of the liver but this was not classifiable as cirrhosis. *Clonorchis* infection may not be a direct cause of cirrhosis or primary

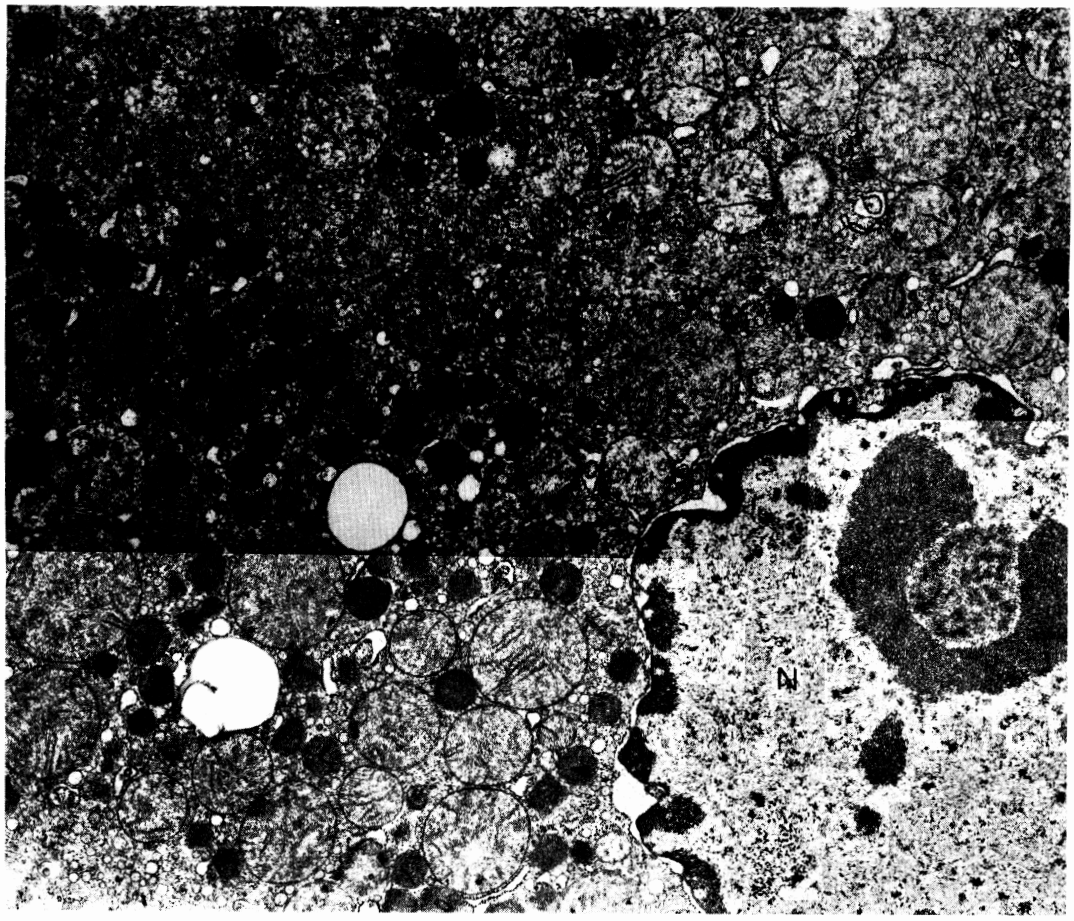

Fig. 4. Ultrastructural findings of liver cell in the rat treated with *Clonorchis* metacercariae plus aflatoxin B_1, at the 28th week separated outer membrane of a nucleus (N) continued to rough endoplasmic reticulum is seen. Irregularity of nuclear membrane is moderate in the degree. There are many electron-dense granules (D) and some vacuoles in cytoplasm (Lead stain, ×12,000).

carcinoma in the liver.

However, the combination of aflatoxin B_1 and *Clonorchis* in the rats of Group III caused much prominent alterations together with pseudolobule formation, cirrhosis and the dysplastic changes of liver cells at later stage. Furthermore, malignant changes showing well differentiated hepatocellular carcinoma were evidenced in two rats at the 28 week. Light microscopically, irregular hyperbasophilic foci composed of dentritic hepatocytes had prominent nucleoli and decrease glycogen, may represent one of the earliest stages in the neoplastic transformation, since these foci are predominant deploid by the cytophotometrical techniques (Gil, *et al.*, 1988). On the other hand, ultrastructural findings showed developed endoplasmic reticulum, irregular size and distribution of gap junctional components, increased dense bodies and free ribosomes. These results suggest that the liver cell foci are potential precursors of hepatic cell carcinoma and may indicate the development of carcinoma (Winston, *et al.* 1988).

In particular, the increased dense bodies in cytoplasm suggest a diminished autophagic sequestration capacity. This may be the most plausible explanation for the decreased rate of proteolysis and assist in liver cell transformation

from neoplastic into true cancer cells (Ahlberg, 1987). Although it has been claimed that spontaneous hepatomas are frequent in a certain strain of rats (Cheever, 1965), it is suggested that the tumor observed in this group were induced due to synergistic action of the two factors as aflatoxin B_1 and *Clonorchis*.

The larvae of *Clonorchis sinensis* provoke the tissue reaction in liver coincidentally, by both mechanical irritation and biological metabolites of worms. The fluke play as an initiator in the histogenesis and aflatoxin B_1 may be responsible for the transformation of dysplastic cells into hepatocellular carcinoma. Aflatoxin B_1 may play a potentiating role as not only a promotor but initiator for the turnover of dysplastic liver cells induced by *Clonorchis* infection, as an initiator (Min, 1986).

These results may suggest that the replicating stages of cells during the proliferative process is sensitive to the action of carcinogens (Craddock, 1973; Fujii & Nakadate, 1977; Thamavit *et al.*, 1978).

The use of promoting agents, following the administration of a chemical being weakly carcinogenic to the liver, can be useful in demonstrating the initiating activity of the chemical (Kitagawa *et al.*, 1979). *Clonorchis sinensis* may increase their susceptibility to action by aflatoxin, and result in increasing the number of carcinogen-susceptible liver cells (Iida 1985). Toxic injuries by aflatoxin and preexisting *Clonorchis sinensis* induced hepatic changes. In Asian countries, the prevalence of both primary carcinoma of the liver and clonorchiasis or opisthorichiasis is high and revealed a higher association of clonorchiasis with cholangiocarcinoma than with hepatocellular carcinoma, but this simultaneous low frequency observed in the Seoul area (Kim, 1984). Thamavit reported that Opisthorchis viverrini associated liver injury and non-specific compensatory regeneration might play an important role in generation of hepatocellular and cholangiocellular carcinomas in man and the continued presence of the parasite might act as a secondary promoting stimulus through chronic increase in cell turnover in the liver. (Thamavit, *et al.* 1988).

Clonorchis sinensis provide a favorable condition for tumorigenesis by aflatoxin B_1.

In conclusion, it is considered in hepatocellular carcinogenesis that the morphological abnormalities in nuclear and nucleolar fine structures are caused by the effect of aflatoxin B_1 and *C. sinensis* may be a specific factor to accelerate the cancer growth from latent foci produced by aflatoxin B_1 exposure.

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