

## Effect of *Clonorchis sinensis* infection and dimethylnitrosamine administration on the induction of cholangiocarcinoma in Syrian golden hamsters

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**Abstract:** The study was carried out to observe the effects of *Clonorchis sinensis* infection on induction of cholangiocarcinoma in Syrian golden hamsters to which 15 ppm dimethylnitrosamine (DMN) solution was administered for 8 weeks. The histopathological changes of the bile duct and liver cells were observed at the 11th week. In six of 8 hamsters (75%) which were treated with DMN and then infected with *C. sinensis*, the livers developed cholangiocarcinoma at 10 weeks after the infestation of *C. sinensis*. The features of cholangiocarcinoma lesions were adenomatous or papillary hyperplasia of the bile duct epithelia showing distinct anaplastic changes with mucinous cell metaplasia and necrotic area. In the hamsters which received either DMN or *C. sinensis* alone, the livers showed only hyperplastic changes of the bile duct epithelial cells. It was suggested that *C. sinensis* infection and DMN administration could be a synergism on the development of cholangiocarcinoma in Syrian golden hamsters.

**Key words:** *Clonorchis sinensis*, cholangiocarcinoma, hamster, dimethylnitrosamine, synergism

### INTRODUCTION

Clonorchiasis is an endemic helminthic disease in southern Korea, Hong Kong, China, Vietnam and Japan (Rim, 1990). *Clonorchis sinensis* has been considered to be a causative factor of cholangiocarcinoma (Hou, 1956; Belamaric, 1973). Cholangiocarcinoma associated with the infection of *C. sinensis* has been reported and a relationship between them has been suggested by many investigators (Hoeppli, 1933; Ch'in *et al.*, 1955; Hou, 1955; Gibson, 1971; Belamaric, 1973; Purtillo, 1976; Kim *et al.*, 1988; Sher, 1989).

Hou (1956) showed the evidence of clonorchiasis in 30 of 200 necropsied cases of primary liver carcinoma. Also, he observed cholangiocarcinoma in cats and dogs infected with *C. sinensis* spontaneously or experimentally (Hou, 1964 and 1965). In an epidemiological study on the relationship between *C. sinensis* infection and primary liver carcinoma, the prevalence rate of cholangiocarcinoma in endemic area, Pusan, appeared to be much higher than that in non-endemic area, Seoul (Chung and Lee, 1976). Infection rate of *C. sinensis* in cholangiocarcinoma cases was higher twice than that in hepatocellular carcinoma cases in man (Kim *et al.*, 1974). According to Chung and Lee (1976), relative risk of *C. sinensis* infection in cholangiocarcinoma cases was found to be about 5

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times greater than that in hepatocellular carcinoma cases.

Belamaric (1973) suggested that there was a cause-to-effect relationship between clonorchiasis and occurrences of cholangiocarcinoma, although the exact mechanism by which *C. sinensis* exerts carcinogenic influence is not clear. Thamavit *et al.* (1978) showed that the hamsters infected with *Opisthorchis viverrini* and later fed DMN for 18 weeks invariably developed cholangiocarcinoma, whereas non-infected control animals fed the same amount of DMN did not develop the tumor. Flavell (1981) and Thamavit (1987) had suggested that there be a synergism between *O. viverrini* infection and DMN on the development of cholangiocarcinoma.

The purpose of the present study is to assess the effect of *C. sinensis* on induction of cholangiocarcinoma in Syrian golden hamsters fed DMN prior to infect with *C. sinensis*.

## MATERIALS AND METHODS

### 1. Collection of metacercariae

*Pseudorashora parva* harbouring the metacercariae of *C. sinensis* were captured in Nakdong-River basin, an endemic area in Korea. The whole muscle of the fish was removed and digested in artificial gastric juice for half an hour at 37°C. The digested materials were filtered with the sieve 0.147 mm in diameter and washed several times with saline. The metacercariae of *C. sinensis* were identified and collected under a stereoscopic microscope.

### 2. Animals and experimental procedure

Syrian golden hamsters (the animal laboratory, College of Medicine, Korea University) aged 3 to 4 weeks were kept 3 in each cage. Forty-eight animals were divided into 4 groups of 12 each as follows. Group I was given 15 ppm DMN (Sigma) in drinking water *ad libitum* for 8 weeks and, 10 metacercariae of *C. sinensis* were infected in each animal at 7 days of the beginning of the experiment. Each animal in Group II received 15 ppm DMN solution for 8 weeks without the worm. Each animal in Group III was infected with 10 metacercariae without DMN. Group IV was non-infected, non-treated control. All

hamsters from each group were killed after 11 weeks of the experiment.

## 3. Histopathological examination

After gross examination for treated animals, the body and resected livers were weighed at the time of necropsy. The livers and kidneys were fixed in 10 % buffered formalin. Paraffin sections were prepared according to the routine histologic techniques. All sections were cut at approximately 5  $\mu$ m and stained with hematoxylin and eosin (HE), and alcian blue and periodic acid-Schiff (AB-PAS) for the microscopic examination.

## RESULTS

### 1. Growth rate and the ratio of liver weight to body weight

As shown in Fig. 1, the average body weight of the animals in Group I was lower than those in Group II, III and IV. Average body weight and percentage of liver weight to body weight in each group at necropsy were shown in Fig. 2. The ratio of liver weight to body weight in Group I and III infected with *C. sinensis* were significantly higher ( $P < 0.001$ ) than that in Group IV.

### 2. Gross finding of the liver

Gross lesions at 11 weeks after the beginnings of the experiment are shown in Table 1. In Group I, most of the livers of hamsters showed pale granular surface and distension of the gall bladder. There was filled with yellowish to reddish brown fluid in

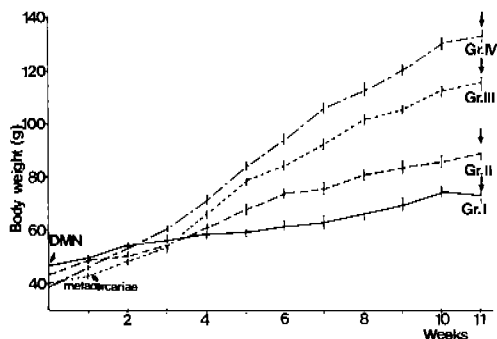


Fig. 1. Changes of body weights in the hamsters.  $\downarrow$ :  $P < 0.001$ , each group is significantly different from control group.

peritoneal cavity of the animals. The livers in 6 of 8 hamsters appeared the protrusive nodularity with solitary, firm, gray-white areas in each lobe. There were gray-white masses measuring 0.5 to 0.7 cm in diameter on the cut surfaces of the liver lobes (Fig. 3). Four hamsters of group 1 died at the 7th, 8th, and 10th week from cannibalism. In Group II, the majority of the livers of hamsters was pale to brown and fragile. Two livers of inspected 12 hamsters showed slight nodularity measuring 0.5 to 1 mm in diameter. In Group III, the

livers of all hamsters showed the cystic distension of gall bladders and swollen figures of the organ.

### 3. Histopathology of the liver

The histopathological lesions of the bile duct of each hamster in the experiment were classified into bile duct hyperplasia, cholangiofibrosis, cholangiofibroma and cholangiocarcinoma according to the criteria by the scheme of Bannasch (1971), Terao (1974), Reddy *et al.* (1977) and Nakajima and Kondo (1989). Table 2 shows the occurrence numbers for the histological diagnosis of the bile duct in the livers in each group. The six of 8 hamsters (75%) in Group I developed cholangiocarcinoma microscopically. All livers of them showed the hyperplasia of bile epithelia, cholangiofibrosis and cystic cholangioma and/or cholangiofibroma. No livers of the hamsters in Group II showed cholangiocarcinoma, but two of 12 hamsters developed the cholangiofibrosis and cholangiofibroma. The livers in Group III showed the changes of bile duct hyperplasia and periductal fibrosis. The livers of the hamsters in Group IV showed normal appearance.

**Bile duct hyperplasia:** The main bile ducts of hamsters infected with *C.sinensis* were markedly dilated with the flukes and showed desquamation and proliferation of the lining cells (Fig. 4). Proliferation of biliary epithelia in

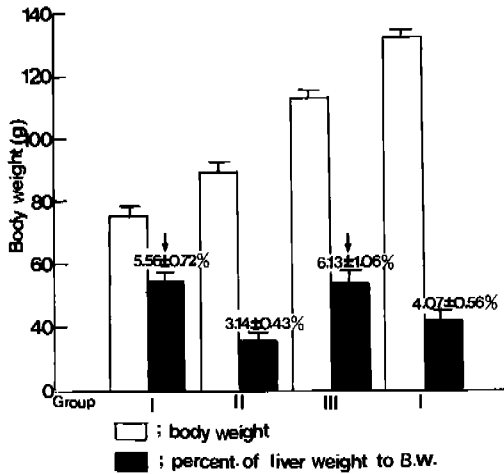


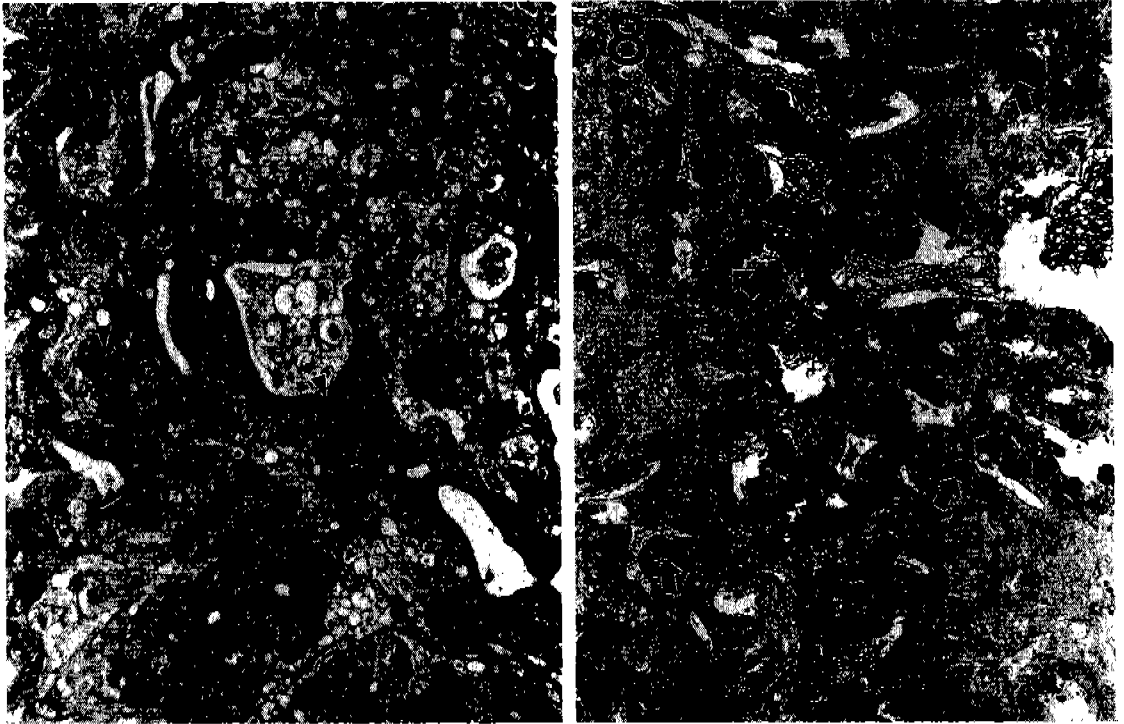
Fig. 2. Average body weights and percent of liver weight to body weight in each group at the 11th week. ↓: Percentages of gr. I and gr. II to gr. IV are significantly high ( $P < 0.001$ ).

Table 1. Gross lesions of the liver of hamsters

Group	Lesions	*LH/IH
I	Granular surface with white spots	8/8
	Severe nodularity (hemorrhagic spots, large white masses with protrusion)	6/8
	White-gray nodule in the parenchyme	6/8
	Large white protrusive mass	2/8
	Ascites	5/8
	Gall bladder distension	8/8
II	Mild nodularity with small white spots	2/12
	Pale color, blunt margination of the hepatic lobules	6/12
	Friable consistency of the liver	6/12
III	Cystic dilatation of the extrahepatic bile duct	12/12
	Swelling of the liver	12/12
IV	No gross lesion	12/12

\*LH/IH: No. of hamsters with the lesion/No. of inspected hamsters.





**Fig. 3.** Cut surface of the liver of a hamster treated with DMN and *C. sinensis* shows gray-white masses (arrow heads) 0.2-0.5 cm in diameter. **Fig. 4.** Microscopic appearance of the liver of a hamster infected with *C. sinensis* shows the dilated bile duct containing the worm (C) and the hyperplasia of bile duct epithelia (arrow). Hematoxylin and eosin (HE) stains ( $\times 100$ ) **Fig. 5.** Microscopic appearance of the liver of a hamster treated with DMN shows adenomatous hyperplasia of bile duct epithelia. HE stains ( $\times 200$ ) **Fig. 6.** Microscopic appearance of the liver of a hamster treated with DMN and *C. sinensis* shows papillary proliferation of the epithelium. HE stains ( $\times 100$ ) **Fig. 7.** In high magnification of Fig. 6, the bile duct shows carcinomatous changes such as irregular stratification, pleomorphism and mitosis (arrow heads) of epithelial cells. HE stains ( $\times 400$ ) **Fig. 8.** Microscopic appearance of cholangiocarcinoma shows a lot of metaplastic goblet cells (arrows) in DMN plus *C. sinensis* group. Alcian blue and periodic acid Schiff stains (AB-PAS) ( $\times 200$ )

hamsters (Group I and a part of Group III) which infected with *C. sinensis* was observed in the portal areas and the hepatic lobules. The epithelia lining the bile duct in Group I showed the pleomorphic features, whereas some of the livers in Group II and III showed intact appearance of the bile ducts. The cuboidal to columnar cells lining the main duct showed pseudostratified arrangement in the hamsters infected with *C. sinensis*. In some lesions with *C. sinensis*, the proliferating epithelial cells formed pseudobiliary lumens.

**Cholangiofibrosis:** Cholangiofibrosis occurred in hamsters in this study was accompanied with bile duct hyperplasia, cholangiofibroma and cholangiocarcinoma. Cholangiofibrosis in the livers of the hamsters in

Group I and a part of Group II was obviously different from that in some of Group III. Cholangiofibrosis induced by treatment of DMN tended to be invasive into the hepatic lobule when compared with the defined periductal fibrosis in the liver of the hamsters infected with *C. sinensis*.

**Cholangiofibroma:** Cholangiofibroma in Group I and II appeared to be well differentiated pattern but some lining cells of the bile duct showed more or less undifferentiated features (Fig. 5).

**Cholangiocarcinoma:** Cholangiocarcinoma was observed only in the hamsters of Group I. The livers in 6 of 8 hamsters (75%) showed markedly nodular and dysplastic changes of bile duct microscopically. The tumor was

designated as mucin secreting cholangiocarcinoma, in which most of the bile ducts were constituted of glandulotubular structures (Fig. 6). There were many metaplastic goblet cells and mucin-filling lumens (Fig. 8). Mitotic figures and hyperchromatic nuclei were common and the neoplastic cells lining the duct showed the pleomorphism and undifferentiated configuration (Fig. 7). In some portal areas, small ductlike cells (Sell, 1983) showed invasive proliferation into hepatic lobules. The livers of the hamsters showed the solitary mass or multiple nodules composed of proliferative ductules and fibrous stroma. These neoplastic nodules were characterized with the various-sized irregular patterns of epithelial cells. Most nodules contained central necrotic areas. There were two lumina within a single acinar, i.e., "gland within gland" patterns. Some lumens of the glands were filled with mucinous materials, desquamated epithelial cells and fragments of inflammatory cells. In addition, peliosis hepatis and megalocytosis with eosinophilic nucleoli were seen.

**Other liver changes:** Besides the above descriptions, the histopathological changes of the livers in the hamsters in each group were

summarized in Table 3. Clear cell foci and hepatocellular necrosis were much prominent in the hamsters of Group I and II treated with DMN. The granulomatous lesion was observed in some hamsters of Group I and III.

### DISCUSSION

The results of this experiment demonstrated a high incidence (75%) of cholangiocarcinomas in the livers of the hamsters which were administered with DMN and then infected with *C. sinensis*. The features of the tumors were characterized with neoplastic hyperplasia of the bile duct having distinctive anaplastic changes with necrotic foci and mucinous metaplasia. Cholangiofibrosis was widely shown in the liver of the hamsters treated with DMN and infected with *C. sinensis*. Cholangiofibrosis observed in the liver of the hamsters seemed as not merely inflammatory reaction but as an autonomous response, and cholangiocarcinoma might be originated from the lining epithelium of the bile duct in the lesion of cholangiofibrosis (Bannasch, 1971; Terao, 1974). In the hamsters infected with *C. sinensis*, there was observed a prominent proliferation of ductular cells. However, we could

**Table 2.** Occurrence numbers for histological lesions of bile duct of hamsters in each group

Group	No. of animals	No. of animals with the histologic lesions			
		Bile duct hyperplasia	Cholangio fibrosis	Cholangio fibroma	Cholangio carcinoma
I	8	8	8	8	6
II	12	3	2	2	0
III	12	10	5	0	0
IV	12	0	0	0	0

**Table 3.** Occurrence numbers of other histopathological changes of the livers in each group

Features\Groups	I	II	III	IV
No. of animals examined	8	12	12	12
Clear cell foci	6	4	0	0
Hepatocellular necrosis	6	6	0	0
Granuloma	2	0	3	0
Mucinous metaplasia	8	2	11	0
Inflammatory cell infiltration	8	2	11	0
Necrosis in nodules	6	0	0	0

not identify the oval cells from proliferating bile duct cells. Sell and Dunsford (1989) proposed that cholangiocarcinoma arose from a stem oval cell. In fact, the changes in portal area seemed to begin with a ductular (oval) cell proliferation (Farber, 1958) which might progressively involved to cholangiofibrotic areas, benign cystic cholangiomas, cholangiofibromas and eventually cholangiocarcinomas (Bannasch and Reiss, 1971; Bannasch and Massner, 1976).

*C. sinensis* is one of the most important helminths in Korea. Cholangiocarcinoma that is concomitant with clonorchiasis in man is relatively common in endemic areas of *C. sinensis* (Kim, 1971; Kim *et al.*, 1974; Chung, 1975; Chung and Lee, 1976). Kim *et al.* (1974) suggested the possibility that clonorchiasis had induced cholangiocarcinoma in man. The relationship between clonorchiasis and cholangiocarcinoma was documented detailedly by Kim (1984). He suggested that the biliary epithelium which was irritated by *C. sinensis* underwent a carcinomatous transformation through a stage of dysplasia.

However, many experimental studies have shown that any animal infected with *C. sinensis* alone never developed the bile duct tumor yet (Wykoff, 1958; Lee *et al.*, 1978a; Lee *et al.*, 1978b; Min and Han, 1985; Chung *et al.*, 1987). The livers of some animals experimentally infected with *C. sinensis* developed adenomatous hyperplasia, periductal fibrosis, inflammatory infiltration and goblet cell metaplasia (Cha, *et al.*, 1991). It was suggested that the *C. sinensis per se* does not provide the sole carcinogenic stimulus leading to malignancy (Flavell, 1982). Although DMN was known to have a potent carcinogenic effect, the hamsters fed DMN alone in our study never developed the tumor. DMN have the genotoxic effect in hepatocarcinogenesis. We suggest that the proliferation of DMN-damaged epithelium of bile duct due to *C. sinensis* infection have influenced on the induction of cholangiocarcinoma. Cell proliferation has often been implicated in the development of cancer with chemicals (Cayama, 1978 ; Farber, 1987). Although the mechanism by which cell proliferation stimulates the induction of tumor is not known, cell proliferation is required for

the replication and the fixing of carcinogen-damaged DNA before repair.

Besides of nitrosamides (Thamavit, 1978, 1987, 1988), aflatoxin B<sub>1</sub> (Chung *et al.*, 1987), 2-AAF (Iida, 1985) have the probability which can produce cholangiocarcinoma in animals experimentally infected with liver fluke. N-nitrosamide derived from secondary amines by nitrosation were commonly found in salted fishes (Fong, 1973), sausage (Williams and Weisburger, 1981), milk and tobacco smoking (Hecht and Hoffman, 1988), and naturally synthetic in mammalian stomach.

Min and Soh (1986) observed the lesion of cholangiocarcinoma in rats which were administered DMN and infected with *C. sinensis*. Chung *et al.* (1987) reported the induction of cholangiocarcinoma in mice treated with aflatoxin B<sub>1</sub> and infected with *C. sinensis*. However, the diagnosis of cholangiocarcinoma is not clear in the photographs presented by Min and Soh (1986) and Chung *et al.* (1987). Also, they did not find out the metastasis of cholangiocarcinoma in their experiments. In fact, it has been difficult to differentiate cholangiocarcinoma from cholangioma and bile duct hyperplasia (Ban-nasch and Reiss, 1971). It is necessary that the biologic features of malignancy (metastasis to other organ, invasion and so on) should be proven in order to confirm the lesion of cholangiocarcinoma. We observed the metastasis of cholangiocarcinoma to other organs at 35 weeks in hamsters which were infected with *C. sinensis* after DMN administration. Induction of cholangiocarcinoma relevant to the infection of *C. sinensis* depends upon a kind of animals. Rats (Min and Soh, 1986) and mice (Chung, 1987) are not susceptible animals in the development of cholangiocarcinoma due to infection of *C. sinensis*.

In this study, Using an appropriate combination of DMN, *C. sinensis* and high susceptible animals, cholangiocarcinomas were induced so fast as 11 weeks in hamsters. This study was shown that *C. sinensis* and DMN had a synergistic effect on the development of cholangiocarcinoma in hamsters. Also, this study suggests that *C. sinensis* infection may act as a promoter of a two-stage carcinogenic process requiring an initiator. It will be most

interesting to assess whether it is able to induce cholangiocarcinoma when an agent is administered to hamsters infected with *C. sinensis*.

Although the induction of cholangiocarcinoma were easily observed in hamsters treated with DMN and *C. sinensis*, the influence of *C. sinensis* infection on the induction of cholangiocarcinoma is not clear.

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=국문초록=

## Dimethylnitrosamine을 투여한 햄스터에서 간흡충감염이 담관암 발생에 미치는 영향

고려대학교 의과대학 기생충학교실<sup>1)</sup> 및 서울대학교 수의과대학 병리학교실<sup>2)</sup>

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간흡충은 담관암 발생의 원인적 요소로 알려졌다. 그러나 간흡충이 담관암을 일으키는 병리기전은 자세하게 알려져 있지 않다. 본 연구는 15 ppm의 dimethylnitrosamine(DMN)을 먹는 물로 투여한 햄스터에 간흡충을 감염시켜 담관암의 발생여부를 알아보고 담관암의 병리조직학적 변화를 관찰하고자 하였다. DMN을 투여하고 간흡충을 감염시킨 햄스터에서 11주 후에 8마리 중 6마리가 육안적 혹은 조직학적으로 담관암(cholangiocarcinoma)의 소견을 보였으며 나머지 2마리는 담관종(cholangiofibroma)으로 진단할 수 있었으나 부분적으로 악성적인 모습도 관찰할 수 있었다. 담관암의 병리조직학적 소견은 담관상피세포가 선종상 혹은 유두상의 증식을 나타내고 높은 이형성을 보였으며 점액세포화생과 괴사소를 보이고 있었다. DMN 만을, 혹은 간흡충만을 투여하거나 감염시킨 햄스터들에서는 담관암이 전혀 발생되지 않았다. 본 실험은 DMN을 투여한 햄스터에 간흡충감염으로 담관암이 발생하는 과정에서 DMN과 간흡충의 협동작용(synergism)을 보여준 것으로 추정한다.

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