Modulation of Dihydroxy-di-n-propylnitrosamine-induced Liver Lesion Development in *Opisthorchis*-infected Syrian Hamsters by Praziquantel Treatment in Association with Butylated Hydroxyanisole or Dehydroepiandrosterone Administration

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The effects of praziquantel coupled with dehydroepiandrosterone (DHEA) or butylated hydroxyanisole (BHA) administration 16 weeks subsequent to dihydroxy-di-n-propylnitrosamine (DHPN) treatment and infection with *Opisthorchis viverrini* (OV) on lesion development in the liver of Syrian hamsters were investigated. Animals were given 80 OV metacercariae and then two i.p. injections of DHPN (500 mg/kg body weight) 4 and 5 weeks thereafter. At week 16, groups received praziquantel (250 mg/kg, i.g.) and were placed on normal diet or diet supplemented with BHA (1%) or DHEA (0.6%) until they were killed at week 24. Histopathological assessment revealed that, whereas antihelminthic treatment alone resulted in a clear reduction in hepatocellular lesion development, effects on cholangiocellular lesions were equivocal. BHA and DHEA, in contrast, were both associated with a significant reduction in frequency of cholangiofibrosis and cholangiocellular carcinoma. The former chemical, however, increased the numbers of liver nodules while the hormone brought about a decrease as well as a shift in the phenotype of the lesions. The results thus indicate that although cholangiocellular lesion development may, unlike generation of hepatocellular nodules, be to a certain extent independent of the continued presence of parasite, it can be influenced by exogenous treatments.

**Key words:** *Opisthorchis viverrini* — Liver cancer — Prevention — Butylated hydroxyanisole — Dehydroepiandrosterone

The great variation in incidence of liver cancer in different parts of the world is thought to be directly related to the presence or absence of predisposing factors, including exposure to viruses, carcinogens, dietary and cultural influences, and in some cases parasites. An especially strong correlation between cholangiocellular type carcinoma development and liver fluke infection in man has been demonstrated in Thailand, Korea and Hong Kong, the parasites causing a variety of proliferative and inflammatory changes in the affected tissues.1–3)

In Thailand, the liver fluke parasite *Opisthorchis viverrini* (OV) is endemic in the Northeast area, man becoming infected by ingestion of intermediate host fresh water fish bearing metacercariae.4) The human environment in this region is also contaminated with aflatoxins and nitrosamines5,6) which are known to be able to induce hepatocellular or cholangiocellular tumor development in a number of rodent species. The extensive use of a fish sauce containing nitrates and other potential carcinogen precursors further suggests that significant exposure to chemical carcinogens may occur via endogenous formation in the gut.7) In the Syrian hamster, infection with liver flukes causes proliferative and inflammatory changes analogous to those observed in man8,9) and results in liver tumors when associated with nitrosamine exposure.10,11)

One approach to elimination of this problem has been removal of the parasite stimulus by a combination of education and chemotherapy, principally involving use of the antihelminthic drug, praziquantel (2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino [2,1-a]isoquinolin-4-one EMBAY 8440, Biltricide®).12) However, one experimental study has shown that despite very effective killing of parasites, the influence on cholangiocellular tumor development may be limited.13) Therefore the present study was undertaken to assess whether administration of the adrenal hormone dehydroepiandrosterone (DHEA) or the synthetic phenolic antioxidant butylated hydroxyanisole (BHA) might exert modulatory effects. The reason

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for the choice of these compounds is that both can act as chemopreventive agents, but by different mechanisms,\textsuperscript{14, 15)} while also exerting carcinogenicity under certain conditions.\textsuperscript{15, 16)}

**MATERIALS AND METHODS**

A total of 140 male Syrian golden hamsters (Armed Forces Research Institute of Medical Science, Bangkok, Thailand) aged 6–8 weeks at the commencement, were kept 5 to a plastic cage in a temperature-controlled room at 24°C. They were fed on stock diet [Zuellig (Gold Coin Mills PTE, Ltd., Singapore)] and tap water \textit{ad libitum} throughout the experimental period. Cyprioid fish harbouring metacercarial cysts were purchased from market places in towns of Northeast Thailand and the muscles from the base of the pectoral fins and tail, as well as the fins and tails themselves, were removed and digested in pepsin solution overnight at 37°C. The whole digested content was then filtered, washed and resedimented several times. Metacercarial cysts of OV were identified under a dissecting microscope and collected.

The carcinogen, dihydroxy-di-n-propylnitrosamine (DHPN), was purchased from Nacalai Chemicals Ltd. (Kyoto), the hormone DHEA from Sigma Chemical Co. (St. Louis, MO) and the antioxidant BHA from Wako Pure Chemicals Industries Ltd. (Osaka).

The animals were divided into 2 main groups, each subdivided into four. All animals initially received 80 OV metacercariae by intragastric intubation, those in groups 1–4 (25 animals each) also being administered 800 mg/kg body weight injections of DHPN, twice, at weeks 4 and 5 (see Fig. 1). Hamsters of groups 5–8 received the saline vehicle alone. At week 16, animals of groups 2–4 and 6–8 were administered a suspension of praziquantel in salad oil, 250 mg/kg body weight via an intragastric tube. Starting at week 16, groups 3 and 7 received dietary supplementation with DHEA (0.6%) and groups 4 and 8 were administered BHA (1%). The animals in the other groups were maintained on basal diet. All surviving animals were killed by exsanguination under ether anesthesia at the end of week 24.

The livers were immediately removed and fixed in 10% buffered formalin for routine embedding in paraffin. Sections cut at 4 \(\mu\)m were stained with hematoxylin and eosin, and proliferative lesions, including areas of cholangiofibrosis and hepatic nodules, were diagnosed as described earlier\textsuperscript{10, 11)} and counted under a microscope. Areas of liver sections analysed were also measured to allow calculation of numbers per unit area. Incidence data were compared statistically using the \(\chi^2\) test and frequency data with the Student’s \(t\) test.

**RESULTS**

Survival in all groups was good, animals killed at week 24 providing the effective numbers. No significant intergroup differences in body weights were observed. Relative liver weights were heavier in the animals treated with OV and the carcinogen than in those receiving OV alone.

![Fig. 1. Experimental protocol: DHEA and BHA effects on reversibility. C, dihydroxy-di-n-propylnitrosamine; OV, \textit{Opisthorchis viverrini} metacercariae; P, praziquantel; S, animals killed.](image-url)
but treatment with praziquantel, DHEA or BHA was without significant influence. In all cases, antihelminthic treatment was effective (less than 2 flukes per liver as opposed to 15 and above in the non-treated cases).

Preneoplastic and neoplastic lesion development was limited to groups 1–4 receiving the DHPN treatment (see Table I for incidence and frequency data). Hepatocellular foci and nodules demonstrating altered morphology were distinguished on the basis of presence or absence of compression. Mixed cell (MC) (mixtures of cells with clear, acidophilic and basophilic morphology) and homogeneously basophilic (B) types were encountered and quantified separately. Cholangiocellular lesions were divided into benign cholangiobiosis and malignant carcinoma categories. Significant decrease in hepatocellular nodules and cholangiobiosis, but not cholangiocellular carcinomas, was associated with praziquantel administration. Preneoplastic lesions of both cell types were further reduced in the hamsters receiving hormonal supplement, with the proportional reduction in nodule development being greater for mixed cell lesions. BHA caused a decrease in cholangiocellular, but not hepatocellular lesion development. When compared with the praziquantel alone group, significant increase in both MC and B foci/nodules was apparent.

**DISCUSSION**

The present study demonstrated that removal of parasites by treatment with the antihelminthic drug praziquantel is not necessarily associated with a reduction in DHPN-initiated tumor development in the intrahepatic bile duct system, in line with earlier findings. However, additional administration of either BHA or DHEA did bring about marked reduction in cholangiocellular lesions. An explanation for the lack of reversal with the anti-parasite treatment alone must remain speculative, but presumably this result reflects a certain autonomy in growth potential of early lesions in the bile ducts. They remain, however, susceptible to exogenous influence. The findings for hepatocellular foci/nodules suggest that continued proliferation is necessary for their appearance, while demonstrating clear modulatory differences between DHEA and BHA.

Our earlier study revealed the 4-week time point after OV infection to be a threshold for significant inhibitory effects of praziquantel on cholangiocellular lesion development in the Syrian hamster model. The antihelminthic agent was administered 12 weeks after DHPN in the present case and again the findings imply that a relatively short exposure to the inflammatory and proliferative influence of liver fluke parasites is sufficient to effectively enhance carcinogenesis in the bile duct tree. While carcinogenicity testing has shown that praziquantel administration is safe and as a preventive measure, its use is clearly advisable to control parasite exposure in affected human beings, additional intervention would appear warranted if a suitable chemopreventive agent is available.

The present results for BHA suggest a positive influence on cholangiocellular lesions but are surprising in terms of the apparent promotion of their hepatocellular counterparts, given the fact that clear inhibitory effects in the rat and hamster have earlier been shown for this compound. One possible explanation for the anomalous findings is that the effects of the antioxidant are altered under the conditions of cirrhosis prevalent with OV infection. Although this question remains to be elucidated, the possibility of an analogy with the effects of β-carotene on lung cancer development in smokers deserves consideration in this respect.

While the underlying mechanisms remain unclear, the adrenal hormone DHEA demonstrated significant inhibition of both hepatocellular and cholangiocellular lesions, in line with that earlier reported for the rat liver. Similar second-stage inhibition by DHEA has previously been described for the skin, colon, lung and thyroid. Since DHEA is known to be an uncompetitive inhibitor of glucose-6-phosphate dehydrogenase, it is conceivable that a
reduction in ribose unit production via the pentose phosphate pathway may be directly involved in inhibition of neoplastic development. This was earlier indicated by the reduced generation of nodules in the Solt-Farber short-term induction brought about by DHEA treatment and its reversal by provision of ribo- and deoxyribonucleosides. However, the hormone could also cause a decrease in the level of NADPH generated by the same pentose phosphate system, and this could be involved. It is, moreover, far from clear whether the short-term inhibition of the key pentose phosphate enzyme actually persists with long-term administration, and in the rat liver many other enzyme species are also affected. The fact that DHEA long-term administration, and in the rat liver many other changes similar to that earlier reported for mice infected with schistosomes appears warranted.

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In conclusion, while praziquantel is very effective for removal of OV parasites in the Syrian hamster liver, the concomitant influence of this antihelminthic agent on tumor development may be only limited. This has an obvious bearing on the human situation, where continued stress must be placed on surveillance of high-risk populations for early cholangiocellular tumors as the most effective approach to control. However, further investigation of the interaction between other exogenous factors and praziquantel in bringing about a reversal of fibrotic changes similar to that earlier reported for mice infected with schistosomes appears warranted.

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