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ORIGINAL ARTICLE

Combination of Praziquantel and Aspirin Minimizes Liver Pathology of Hamster *Opisthorchis viverrini* Infection Associated Cholangiocarcinoma

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Abstract Opisthorchiasis is one of the major risk factors for cholangiocarcinoma (CCA) in northeastern Thailand. An effective drug for killing this parasite is praziquantel. Recently, several reports have shown that with frequent use, praziquantel may itself be a CCA risk and can cause liver cell damage from an immunopathological response after parasite death. Aspirin has many properties including anti-inflammation and anti-cancer. Therefore, we use of aspirin (*As*) and praziquantel (Pz) to improve hepatobiliary system function in hamsters infected with *Opisthorchis viverrini* (OV) and or administered *N*-nitrosodimethylamine (ND). Livers of OVND*As*Pz, appeared healthy macroscopically, suggesting slow progression of cholangiocarcinoma evident by extent of fibrosis and bile duct cell proliferation was less than OVND although aggregations of inflammatory cells remained. Proliferating cell nuclear antigen (PCNA), cytokeratin 19 (CK19), and cancer antigen (CA19-9) staining were strongly positive in OVND, but were only slight in OVND*As*. Moreover, OVND*As*Pz, appeared a few inflammatory infiltrations, bile duct proliferation, fibrosis and CCA area than the OVND*As* group. Thirty seven point five percent of hamster in this group could not develop CCA. These findings suggest that using aspirin combination with praziquantel treatment can improve the hepatobiliary system after *O. viverrini* infection and reduce the risk of CCA.

Keywords Praziquantel · Aspirin · *Opisthorchis viverrini* · *N*-nitrosodimethylamine · Cholangiocarcinoma · Syrian hamster

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Introduction

Opisthorchiasis, a human liver fluke infection caused by *Opisthorchis viverrini* is widely distributed in many parts of Southeast Asia, including northeastern Thailand [1]. This parasite is responsible for many hepatobiliary diseases including cholangiocarcinoma (CCA) [2]. Praziquantel is an effective drug for treatment of opisthorchiasis. After treatment with praziquantel, not only are the parasites killed, but the immune response by the host, elicited by the presence of dead worms, results in increased liver cell damage by free radicals from inflammatory cells, and consequently a higher risk of CCA [2, 3]. Treatment of CCA remains a problem with a high mortality rate because patients frequently come to the hospital very late. Researchers seeking ways to prevent CCA have focused on the use of plant extracts or drugs whose properties inhibit inflammation [4–6] and generally study these in vitro, or in a hamster model [7–10]. Aspirin is a non-steroidal anti-inflammatory drug found to contribute to cancer prevention through induction of apoptosis in tumor cells [11–13]. Several reports found that use of low doses of aspirin reduced the risk of cancer development, i.e. breast, colorectal, and pancreatic cancer [14–17]. We therefore determined the effects of aspirin

and aspirin combination with praziquantel on hamster liver pathology.

Materials and Methods

Animals

Female Syrian hamsters, 6 to 8 weeks old, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were divided into 6 groups, 8 hamsters per group for study of opisthorchiasis pathology and CCA development following our previous studies [18, 19] : i) administered *N*-nitrosodimethylamine (ND) for induction of CCA; ii) administered ND and aspirin (ND*As*) to demonstrate the anti-CCA effect of aspirin; iii) infected with *O. viverrini* and administered ND (OVND), which is our CCA model; iv) infected with *O. viverrini* and administered ND and aspirin (OVND*As*) to demonstrate any chemopreventive effect of aspirin; v) infected with *O. viverrini* and administered ND and praziquantel (OVND*Pz*) to determine the extent of CCA development post praziquantel treatment; and vi) infected with *O. viverrini* and administered ND, aspirin and praziquantel (OVND*As*Pz) to determine the extent of CCA development after combination of both drugs (Fig. 1). All hamsters were sacrificed at 120 days of the experiment, and photographs were taken for comparison of the gross anatomy of the livers. All work was conducted with the approval of the Khon Kaen University Animal Ethics Committee (AEKKU34/2553).

Preparation of Metacercariae and *O. viverrini* Infection

Naturally infected cyprinid fish were captured from a freshwater reservoir in an endemic area of Khon Kaen, northeastern Thailand. The fresh fish were digested by pepsin-HCl at 37 °C for 1 h, filtered and sedimented with normal saline in a sedimentation jar. *Opisthorchis viverrini* metacercariae were clearly seen under a dissecting microscope. Each hamster was infected with 50 metacercariae by intragastric intubation.

Preparation of *N*-Nitrosodimethylamine for Inducing Cancer

ND (12.5 ppm in drinking water) was administered every day during 30 days to 60 days of the experiment (Modified from Thamavit et al. [20]).

Praziquantel Administration

Praziquantel was purchased from Medicpharma Co., Ltd., Bangkok, Thailand. For the purpose of killing the parasites, 400 mg/kg of praziquantel were orally administered to the assigned hamsters 30 days post *O. viverrini*-infection.

Aspirin Administration

Acetylsalicylic acid powder (Sigma-Aldrich, St. Louis MO, USA) was dissolved and sprayed onto animal food pellets. Food-Aspirin pellets were given every day until sacrifice. Each hamster received approximately 30 mg/kg of aspirin per day (Modified from Miliaras et al. [21]).

Animal Sacrifice and Specimen Collection

Hamsters were euthanized with an overdose of diethyl ether. The liver, gallbladder and extrahepatic bile duct were carefully dissected following the methods described previously [18, 22]. The liver was fixed in 10 % buffered formalin solution

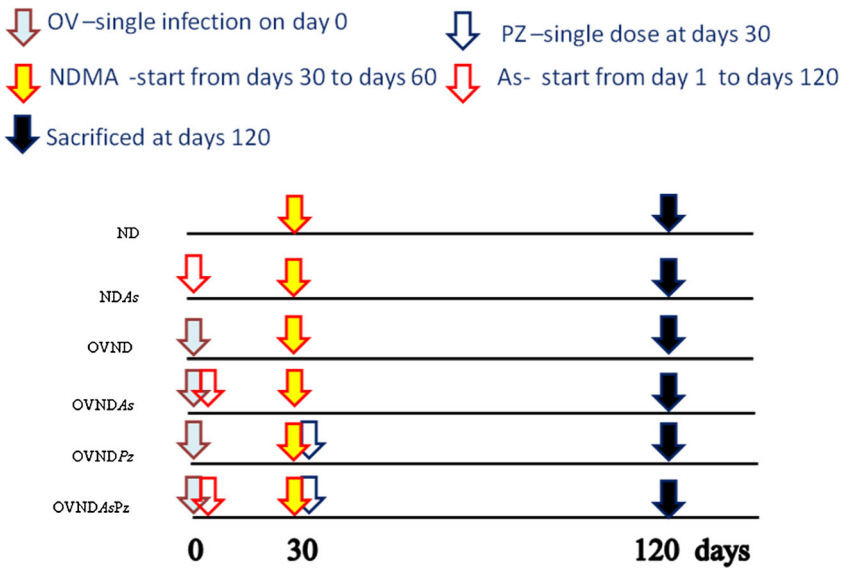

Fig. 1 Scheme of the treatments to animal groups

for histopathological study. Specimens were processed for histology in a conventional manner and sections stained with hematoxylin and eosin (H&E), Sirius red and immunohistochemical reagents.

Hematoxylin and Eosin Staining

Tissue sections were deparaffinized in xylene, rehydrated through a descending alcohol series, washed with distilled water and stained with Harris's hematoxylin followed by eosin. Finally, the sections were dehydrated through an alcohol series, cleared in xylene, and mounted on slides using Permount resin. The liver sections were observed and digitized under a light microscope (Olympus BX51; Tokyo, Japan) for pathological grading.

Score: Hepatic tissue inflammation: 0 = minimal/no liver tissue inflammation, 1 = mild (inflammatory cell <1/3 in liver tissue areas), 2 = moderate (increase in inflammatory cells on 1/3—2/3 of liver tissue areas), 3 = severe (dense packing of inflammatory cell >2/3 of liver tissue areas); Lobular inflammation: 0 = minimal/no portal inflammation, 1 = mild (inflammatory cell <1/3 of periportal areas), 2 = moderate (increase in inflammatory cells on 1/3—2/3 of periportal areas), 3 = severe (dense packing of inflammatory cell >2/3 of periportal areas); New bile duct proliferation and CCA area: 0 = no new bile duct proliferation and CCA area in liver tissue, 1 = a few new bile duct proliferation but no CCA area in liver tissue, 2 = moderate new bile duct proliferation ± a few CCA area in liver tissue, 3 = a lot of new bile duct proliferation + CCA area 15–30 % in liver tissue, 4 = a lot of new bile duct proliferation + CCA area 30–50 % in liver tissue, 5 = a lot of new bile duct proliferation + CCA area more than 50 % in liver tissue. N, number of hamster (Modified from Wonkchalee et al. [10]).

Sirius red Staining to Demonstrate Fibrosis

Liver sections were stained with Sirius red. In brief, deparaffinized and rehydrated sections were stained with hematoxylin for nuclear staining and then stained with a saturated aqueous solution of picric acid containing 0.1 % Sirius Red (Sigma-Aldrich) for 1 h, washed with acidified water, dehydrated in an ascending series of ethanol and cleared in xylene, and mounted. Collagen fibers in the connective tissues were stained red. The liver sections were observed and digitized under a light microscope (Olympus BX51; Tokyo, Japan) for grading fibrosis. Score: Hepatic tissue fibrosis: 0 = no fibrosis, 1 = short fibrous septa ± a few fibrosis area in liver tissue, 2 = occasional P-P bridge with fibrosis area 15–30 % in liver tissue, 3 = marked bridge with fibrosis area 15–30 % in liver tissue, 4 = marked bridge with fibrosis area > 50 % in liver tissue; Portal fibrosis: 0 = no fibrosis or a few fibrous expanded not surrounding the portal tract, 1 = fibrous expansion of some portal area surrounding the portal tract ±short fibrous septa, 2 = fibrous expansion of most portal area surrounding the portal tract ±short fibrous septa, 3 = thickness fibrous expansion of some portal area surrounding the portal tract ± short fibrous septa, 4 = thickness fibrous expansion of most portal area surrounding the portal tract ± short fibrous septa (Modified from Wonkchalee et al. [10]).

Immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA), Cytokeratin 19 (CK19) and Cancer Antigen 19—9 (CA19-9)

To determine the effects of aspirin supplementation on CCA development, the expression of PCNA (central to both DNA replication and repair), CK19 (widely related to the study of development of the biliary tree) and CA 19—9 (tumor marker) in the liver tissue sections were immunohistochemically examined. After antigen retrieval (boiled in 5 M sodium citrate buffer pH 6 for 5 min), tissue sections were blocked for endogenous peroxidase activity. Nonspecific background was blocked using 5 % skim milk. Specimens were incubated for 1 h at 37 °C with mouse monoclonal antibody for PCNA (Novocastra Laboratories, Newcastle upon Tyne, UK), rabbit polyclonal antibodies for CK19 (Abcam, Cambridge MA, USA) and mouse monoclonal antibody for CA19-9(Abcam, Cambridge MA, USA) at a dilution of 1:300, and then washed three times with phosphate-buffered saline (PBS). A negative control study was performed under the same conditions using normal serum instead of the primary antibody. EnVision + system-HRP labeled polymer anti-mouse and anti-rabbit antibodies (Dako, Glostrup, Denmark) were used for secondary antibody. Specimens were incubated for 1 h, and then developed for color with diaminobenzidine or AEC as a visual marker. Digital photomicrographs of the stained sections were taken with an Olympus BX51 microscope. Immunohistochemical stainings were evaluated by grading score. Score; 0 = no or a few positive staining area (<10 %), 1 = positive staining area < 30 %, 2 = positive staining area 30–60 %, 3 = positive staining area > 60 % (Modified from Muerkoster et al. [23]).

Statistical Analysis

Statistics were analyzed using SPSS version 19.0 statistical software (SPSS, USA). Differences in values were considered statistically significant when $P < 0.05$.

Results

In this study, 48 hamsters were divided into 6 groups (8 animals/group) for investigation of the effects of aspirin and/or praziquantel treatment. Hamsters treated with aspirin (ND*As*,

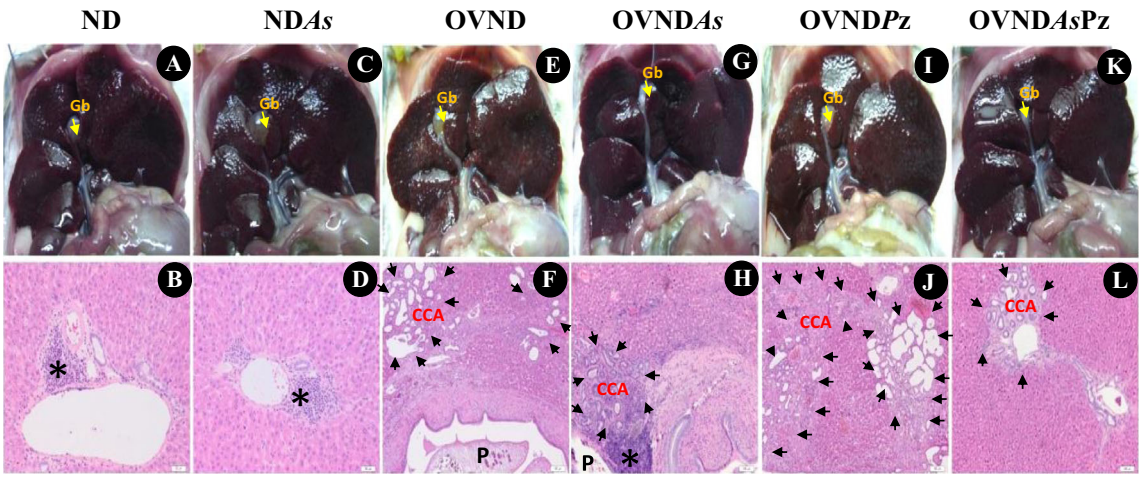

Fig. 2 Representative gross and histopathology in Syrian hamsters administered NDMA (ND, **a-b**), administered NDMA and aspirin (ND*As*, **c-d**), infected with *O. viverrini* and administered NDMA (OVND,**e-f**), infected with *O. viverrini*, administered NDMA and aspirin (OVND*As*, **g-h**), infected with *O. viverrini*, administered NDMA and praziquantel (OVND*Pz*, **i-j**), and infected with *O. viverrini*, administered NDMA, aspirin and praziquantel (OVND*As*Pz, **k-l**). *Gb* gallbladder, *P* parasite, *Star* inflammatory cell, *CCA and arrow* cholangiocarcinoma area

OVND*As*, OVND*As*Pz) had a 100 % survival rate until the end of the experiment, but other groups showed decreased rates of survival: 87.5 % for ND and OVND, and 75 % for OVND*Pz*.

Gross and Histopathological Appearance at 120 Days

Liver surface and color of both ND and ND*As* groups (Fig. 2a, c) were red brownish and with no nodular formation, which appeared similar to the normal condition [19]. Dilation of extrahepatic bile ducts and opaque gallbladder were observed in the infected groups OVND and OVND*As* (Fig. 2e, g), but not in the praziquantel-treated groups (Fig. 2i, k). Table 1 shows histopathological grading correlated with the gross results. In Syrian hamsters administered ND only (Fig. 2b) and administered ND and aspirin (Fig. 2d), there were aggregations of inflammatory cells in the portal triads. A large CCA area can be observed in the OVND (Fig. 2f) and OVND*Pz* (Fig. 2j) groups, while a small area of CCA can be observed in both aspirin-treated groups, OVND*As* (Fig. 2h) and OVND*As*Pz (Fig. 2l).

Table 1 Histological grading criteria of liver biopsy by microscopic observation

Histopathology	Score	ND N = 7 % (N)	ND*As* N = 8 % (N)	OVND N = 7 % (N)	OVND*As* N = 8 % (N)	OVND*Pz* N = 6 % (N)	OVND*As*Pz N = 8 % (N)
Hepatic tissue inflammation	0						
	1	85.7 (6)	87.5 (7)	14.3 (1)		33.3 (2)	50.0 (4)
	2	14.3 (1)	12.5 (1)	57.1 (4)	62.5 (5)	50.0 (3)	50.0 (4)
	3			28.6 (2)	37.5 (3)	16.7 (1)	
Lobular inflammation	0	14.3 (1)					
	1	71.4 (5)	50.0 (4)			16.7 (1)	50.0 (4)
	2	14.3 (1)	50.0 (4)	57.1 (4)	25.0 (2)	50.0 (3)	37.5 (3)
	3			42.9 (3)	75.0 (6)	33.3 (2)	12.5 (1)
New bile duct proliferation and CCA area	0	57.1 (4)	62.5 (5)				12.5 (1)
	1	28.6 (2)	25.0 (2)			33.3 (2)	25.0 (2)
	2	14.3 (1)	12.5 (1)	42.9 (3)	75.0 (6)	16.7 (1)	37.5 (3)
	3			14.3 (1)	25.0 (2)	33.3 (2)	25.0 (2)
	4			28.5 (2)		16.7 (1)	
	5			14.3 (1)			

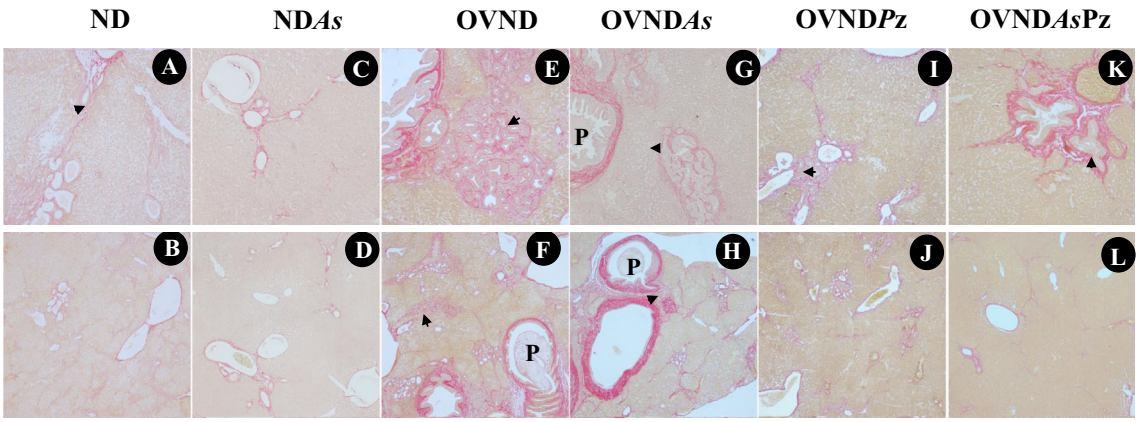

Fig. 3 Representative fibrosis using sirius red in Syrian hamster administered with NDMA (ND, **a-b**), administered with NDMA and aspirin (ND*As*, **c-d**), infected with *O. viverrini*, administered with NDMA (OVND, **e-f**), infected with *O. viverrini*, administered with NDMA and aspirin (OVND*As*, **g-h**), infected with *O. viverrini*, administered with NDMA and praziquantel (OVND*Pz*, **i-j**) and infected with *O. viverrini*, administered with NDMA, aspirin and praziquantel (OVND*As*Pz, **k-l**). *P* parasite, *Arrow* positive staining

Effect of Aspirin on Fibrosis in a Hamster CCA Model

Figure 3 shows fibrosis in hepatic tissue and surrounding hepatic bile ducts in all groups, but to a different degree. In the ND group, some fibrosis was observed at the bile ducts of the portal triad (Fig. 3a) and sub-scapular area (Fig. 3b), which was similar to the ND*As* group (Fig. 3c, d). The OVND*As* group (Fig. 3g, h) and praziquantel treatment in the OVND*Pz* group (Fig. 3i, j) exhibited reducing of fibrosis compared to the OVND group (Fig. 3e, f) as shown in Table 2. Interestingly, administration of praziquantel subsequent to aspirin treatment in the OVND*As*Pz group (Fig. 3k, l) resulted in greater reduction of fibrosis with statistic significantly difference ($P < 0.05$). The liver fibrosis has shown in Table 2.

Immunohistochemistry for Cytokeratin 19 (CK19)

CK19 staining results were correlated with histopathology. Figure 4 shows positive staining for CK19 at the bile ducts in the ND (Fig. 4a, b), ND*As* (Fig. 4c, d), OVND (Fig. 4e), OVND*As* (Fig. 4g) and OVND*As*Pz (Fig. 4k, l) groups, but to a different degree based on the area or number of bile ducts. Not only bile duct cytoplasm but also CCA cytoplasm was positive, especially in the OVND (Fig. 4f), OVND*Pz* (Fig. 4i, j) and OVND*As* (Fig. 4h) groups. Positive staining in OVND*As*Pz group was significantly decreased when compared with OVND group ($P < 0.05$) as shown in Table 3.

Immunohistochemistry for Cancer Antigen 19–9 (CA19-9)

Immunohistochemistry for CA19-9 was used to confirm the CCA area in the liver tissue. Figure 5 shows the positive staining of CA19-9 in the cytoplasm of CCA area. Positive staining was observed in the liver tissue of OVND, OVND*Pz* and OVND*As* groups with different degree. The largest

Table 2 Fibrosis grading criteria of liver biopsy by microscopic observation

Histopathology	Score	ND N = 7 % (N)	ND*As* N = 8 % (N)	OVND N = 7 % (N)	OVND*As* N = 8 % (N)	OVND*Pz* N = 6 % (N)	OVND*As*Pz N = 8 % (N)
Hepatic tissue fibrosis	0						
	1	85.7 (6)	62.5 (5)	14.3 (1)	25.0 (2)	33.3 (2)	62.5 (5)
	2	14.3 (1)	37.5 (3)	28.6 (2)	62.5 (5)	33.3 (2)	25.0 (2)
	3			42.8 (3)	12.5 (1)	33.3 (2)	12.5 (1)*
	4			14.3 (1)			
Portal fibrosis	0						
	1	85.7 (6)	87.5 (7)				37.5 (3)
	2		12.5 (1)	28.6 (2)	25.0 (2)	50.0 (3)	37.5 (3)
	3	14.3 (1)		28.6 (2)	37.5 (3)	33.3 (2)	25.0 (2)*
	4			42.9 (3)	37.5 (3)	16.7 (1)	

N number of hamster, * $P < 0.05$ compared with OVND group

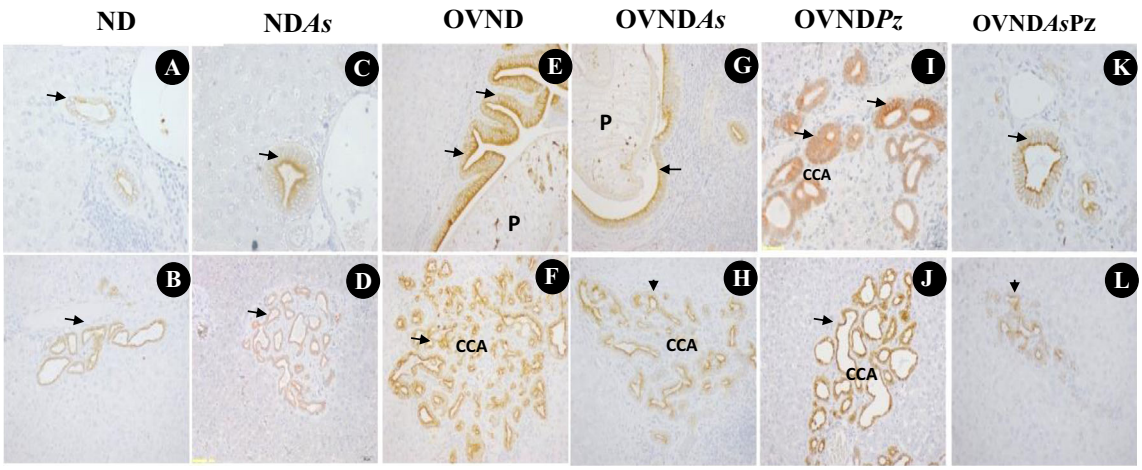

Fig. 4 Representative immunostaining for Cytokeratin19 (CK19) in Syrian hamster administered with NDMA (ND, **a-b**), administered with NDMA and aspirin (ND*As*, **c-d**), infected with *O. viverrini*, administered with NDMA (OVND, **e-f**), infected with *O. viverrini*, administered with NDMA and aspirin (OVND*As*, **g-h**), infected with *O. viverrini*, administered with NDMA and praziquantel (OVND*Pz*, **i-j**) and infected with *O. viverrini*, administered with NDMA, aspirin and praziquantel (OVND*As*Pz, **k-l**). *P* parasite, *Arrow* positive staining, *CCA* cholangiocarcinoma

positive staining area was OVND and subsequence to OVND*Pz* and OVND*As* respectively. Negative staining was observed in ND, ND*As* and OVND*As*Pz groups.

Immunohistochemistry for Proliferating Cell Nuclear Antigen (PNCA)

Figure 6 shows positive staining for PNCA in the nuclei of bile ducts in all groups. Positive staining was most conspicuous in the OVND group (Fig. 6e, f), followed by ND (Fig. 6a, b)the OVND*As* (Fig. 6g, h), OVND*Pz* (Fig. 6i, j), OVND*As*Pz (Fig. 6k, l), ND (Fig. 6a, b), and ND*As* (Fig. 6c, d) groups, respectively. Positive staining in OVND was significantly higher than OVND*As* and OVND*As*Pz ($P < 0.05$), as shown in Table 3.

Discussion

Our present study is the first report to find that aspirin reduces liver fibrosis and can inhibit CCA development and tumor size in the hamster CCA model, especially in the group of praziquantel treatment evident by histopathological observation through H&E, Sirius red staining and immunochemical staining for CA19-9, CK 19 and PCNA. Moreover, we demonstrated that CCA could be observed in the group that had been cleared of infection by administration of praziquantel.

It is well established that *O. viverrini* is a type I carcinogen [24]. Human CCA is most prevalent in northeast Thailand, where there is the highest incidence of *O. viverrini* infection [25–27]. Although current *O. viverrini* infection is very rare in CCA patients, most CCA patients have a history of *O. viverrini* infection and consumption of raw fish [28–30]. Several previous studies have attempted to use anti-cancer drugs or plants for prevention or treatment of CCA in a hamster model but there were none [18, 19]. This may be caused by the remaining the partial obstruction form parasite which cause sclerosing cholangitis, is a risk factor of CCA.

Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug (NSAID) that has long been used to treat pain, inflammation, fever and prevention of cancer. The present study found that administration of praziquantel and aspirin increased the survival rate of infected animals. Similarly, previous human trials and cohort studies showed that administration of aspirin three or more times weekly for at least a year

Table 3 The expression of cytokeratin 19 (CK19) and proliferating cell nuclear antigen (PCNA) in liver tissue as assessed with immunohistochemistry

Immunohistochemical target	ND	ND*As*	OVND	OVND*As*	OVND*Pz*	OVND*As*Pz
	N = 7	N = 8	N = 7	N = 8	N = 6	N = 8
CK19	1.29 ± 0.18	1.25 ± 0.16	2.57 ± 0.20	2.25 ± 0.16	2.00 ± 0.26	1.50 ± 0.27*
PCNA	0.90 ± 0.23	0.70 ± 0.21	2.60 ± 0.16	2.00 ± 0.21*	1.80 ± 0.20*	1.60 ± 0.22*

The data represent in mean ± S.E.M. N number of hamster, * $P < 0.05$ compared with OVND group

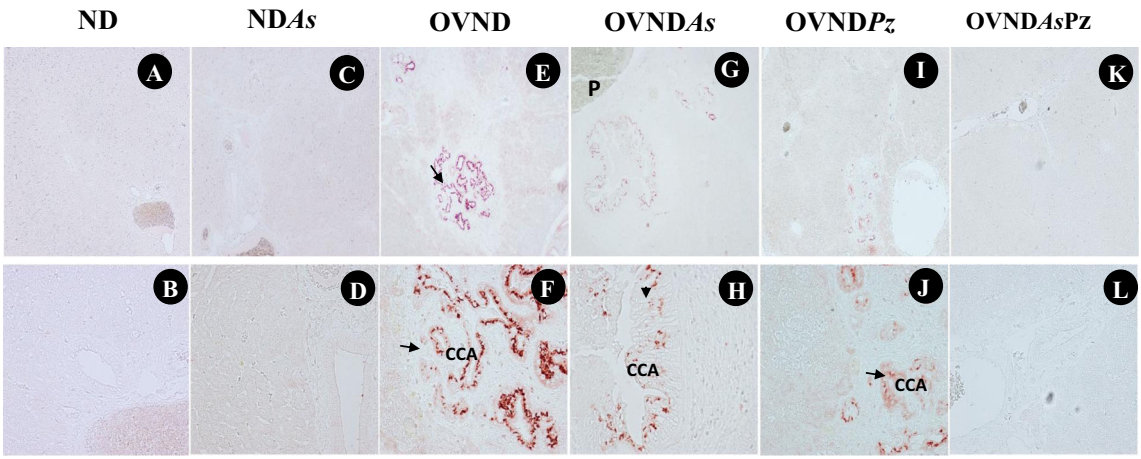

Fig. 5 Representative immunostaining for cancer antigen 19–9 (CA19-9) in Syrian hamster administered with NDMA (ND, **a-b**), administered with NDMA and aspirin (ND*As*, **c-d**), infected with *O. viverrini*, administered with NDMA (OVND, **e-f**), infected with *O. viverrini*, administered with NDMA and aspirin (OVND*As*, **g-h**), infected with *O. viverrini*, administered with NDMA and praziquantel (OVND*Pz*, **i-j**) and infected with *O. viverrini*, administered with NDMA, aspirin and praziquantel (OVND*As*Pz, **k-l**). *P* parasite, *Arrow* positive staining, *CCA* cholangiocarcinoma

can reduce the risk of breast cancer [31] and postmenopausal breast cancer [32]. Another study of breast cancer patients treated with daily doses of aspirin found reduced rates of metastasis and death [33, 34]. Several case–control studies of colorectal cancer patients administered low doses of aspirin showed an association with decreased risk of colorectal cancer [35–37] and pancreatic cancer [38, 39]. Aspirin treatment groups (OVND*As* and OVND*As*Pz) exhibited smaller CCA areas compared to untreated groups (OVND and OVND*Pz*) evident by histopathological result (Fig. 2, Table 1), CK19 (Fig. 4) and CA19-9 (Fig.5). Several reports have shown that CA19-9 is a tumor marker and prognostic marker for several cancers [40, 41] including CCA [42, 43]. Moreover, the PCNA result demonstrated that aspirin treatment groups had a fewer area of expression compared to untreated groups. These data support the findings in previous studies which observed similar pathological appearance (high PCNA and CK19 expression) in CCA development [18, 44]. Moreover, the results of the present study indicate that a combination of praziquantel and aspirin retard liver fibrosis (Fig. 3l and Table 2), which is in accordance with previous reports on liver fibrosis in rats [45] and hypertrophic scar formation in a rabbit ear fibrotic model [46]. The present findings suggest that praziquantel administration subsequent to low doses of aspirin treatment could inhibit and/or prevent CCA development.

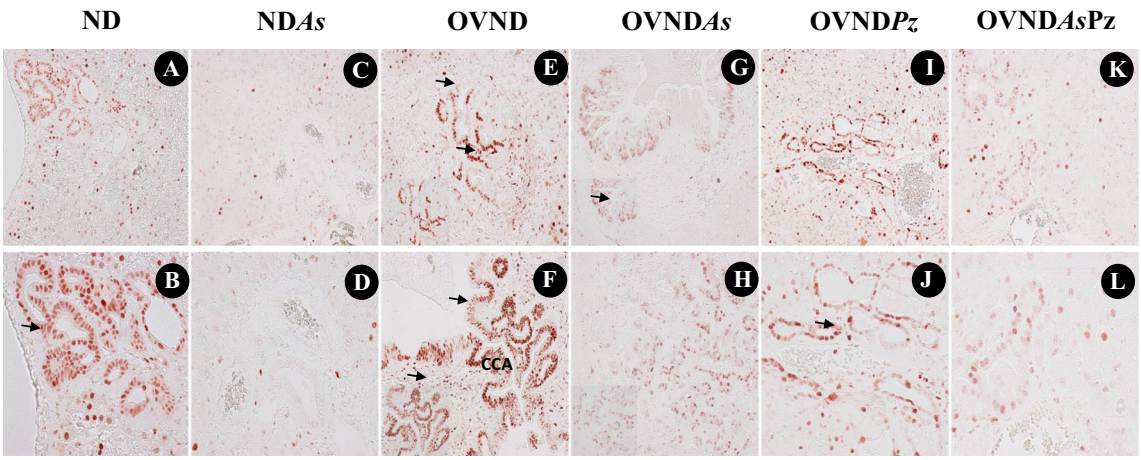

Fig. 6 Representative immunostaining for proliferating cell nuclear antigen (PCNA) in Syrian hamster administered with NDMA (ND, **a-b**), administered with NDMA and aspirin (ND*As*, **c-d**), infected with *O. viverrini*, administered with NDMA (OVND, **e-f**), infected with *O. viverrini*, administered with NDMA and aspirin (OVND*As*, **g-h**), infected with *O. viverrini*, administered with NDMA and praziquantel (OVND*Pz*, **i-j**) and infected with *O. viverrini*, administered with NDMA, aspirin and praziquantel (OVND*As*Pz, **k-l**). *Arrow* positive staining, *CCA* cholangiocarcinoma

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