

ROLE OF MATRIX METALLOPROTEINASE (MMP)-2 AND MMP-9 IN THE PATHOGENESIS OF HAMSTER OPISTHORCHIASIS: EFFECT OF PRAZIQUANTEL TREATMENT

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Abstract. To clarify the role of matrix metalloproteinases (MMPs) in the pathogenesis of opisthorchiasis and the effect of the treatment with praziquantel (an anti-parasitic drug), we examined MMP-2 and MMP-9 activities, using gelatin zymography and hydroxyproline assay, in hamsters infected with *O. viverrini* for 21 days (acute) and 4 months (chronic) compared with those in infected hamsters following praziquantel treatment for 1, 3, and 6 months. The activities of these MMPs were higher in the hamsters with acute infection than in those with chronic infection after drug treatment. The plasma hydroxyproline level increased in a time-dependent manner and was correlated with the thickening of periductal fibrosis in *O. viverrini*-infected hamsters. Praziquantel treatment decreased the plasma hydroxyproline concentration in *O. viverrini*-infected hamsters. The profile of the hydroxyproline level of the acute infection group was lower than that of the chronic infection group, suggesting MMP-2 and MMP-9 are associated with resorption of the periductal fibrosis triggered by *O. viverrini* infection, but that praziquantel treatment may cause a decrease in tissue resorption of periductal fibrosis.

INTRODUCTION

Opisthorchiasis, caused by infection with *Opisthorchis viverrini*, is the major risk factor for cholangiocarcinoma (CCA) development (IARC, 1994) and is still a major health problem in northeast Thailand. *O. viverrini* infection induces several pathological changes (hyperplasia, bile duct proliferation, granulomatous formation, and periductal fibrosis), in both human and animal studies (Bhamarapravati *et al*, 1978; Harinasuta and Harinasuta, 1984). The severity of the disease is dependent on the duration and frequency of infection (IARC, 1994). Periductal fibrosis is the most prominent histological feature during chronic infection in the animal model (Pinlaor *et al*, 2004) and it persists after one-week praziquantel treatment (Pinlaor *et al*, 2006). Irreversible fibrosis may result in primary sclerosing cholangitis

(PSC), which leads to CCA development (Boberg *et al*, 2002). Therefore, the molecular mechanism of fibrosis in opisthorchiasis-associated cholangiocarcinogenesis needs to be elucidated.

Periductal fibrosis results from a relative imbalance between synthesis and degradation of extracellular matrix (ECM) proteins that play important roles in the pathogenesis of several diseases including carcinogenesis. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that specifically degrade ECM component (Visse and Nagase, 2003). The MMPs are produced as zymogens (pro-MMPs) that must be removed during activation. Proteolytic degradation of ECM is an essential feature of tissue remodeling of connective tissue under physiological and pathological conditions (Gomez *et al*, 1997). Therefore, an analysis of the role of various MMP activities may provide basic information in opisthorchiasis-associated CCA.

The gelatinases MMP-2 and MMP-9 are capable of digesting components of connective tissue matrix and several types of collagen,

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which is a major component of the ECM. MMP-2 and MMP-9 contribute to tissue injury in experimental models, are strongly associated with the formation of granulomatous fibrosis in angiostrongyliasis (Hsu *et al*, 2005), and are associated with the recovered stage in CCl₄-induced tissue damage (Knittel *et al*, 2000). Although the role of MMPs in opisthorchiasis is not clear, these MMPs appear to contribute to the fibrosis induced by *O. viverrini* infection.

The expressions of MMP-2 and MMP-9 in the plasma of hamsters infected with *O. viverrini* were analyzed using gelatin zymography in order to elucidate the tissue remodeling in opisthorchiasis, before and after drug treatment in the variably induced injuries, between acute and chronic infection. Hydroxyproline, a major component of collagen, in the plasma was assessed by spectrophotometer. The periductal fibrosis was studied by staining paraffin sections with Masson trichrome.

MATERIALS AND METHODS

Experimental animals

Four- to six-week-old male, Golden hamsters were housed under conventional conditions and fed a stock diet and given water *ad libitum*. Metacercariae of *O. viverrini* were isolated from cyprinoid fish under a dissecting microscope after artificial pepsin digestion as described by Pinlaor *et al* (2004). Five hamsters were put in each group then infected by oral inoculation with 50 *O. viverrini* metacercariae. Animals were sacrificed on days 21, 51, 111, 120, 150, 201, 210, and 300 post-infection. In addition, normal hamsters were used as a control group and sacrificed at these time points.

In the praziquantel treatment groups, after the hamsters had been infected with the metacercariae for 21 days (for the acute phase) and 4 months (for the chronic phase), a single dose of praziquantel (Biltricide, Bayer) 400 mg/kg body weight, suspended in 2% chemophore EL (Sigma, St Louis, MO) was given orally. The animals were sacrificed 1, 3, and 6 months after receiving this treatment. The control group of *O. viverrini*-infected hamsters received only 2% chemophore solution.

Samples at the hilar region of the liver and EDTA blood were collected. After centrifugation at 5,000g for 5 minutes, plasma was collected and stored at -80°C until assayed.

The Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand approved this study.

Gelatin zymography

Hsu *et al* (2005), described substrate-specific zymography for determination of gelatinolytic activities of MMP-2 and MMP-9 in plasma. Briefly, 5 µl of plasma was diluted in 20 µl Tris-buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl and 5 mM CaCl₂) and 25 µl sample buffer [500 mM Tris-HCl, pH 6.8, 17.4% (w/v) glycerol, 4% sodium dodecylsulphate and 0.01% bromophenol blue]. An aliquot of this solution (6 µl) was loaded onto the 7.5% (mass/volume) sodium dodecylsulphate-polyacrylamide gels co-polymerized with 0.1% gelatin (Sigma). Stacking gels were 4% (mass/volume) polyacrylamide gels and did not contain gelatin substrate.

Gel electrophoresis was performed using a mini-gel system apparatus (Bio-Rad). Electrophoresis was performed in running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS) at room temperature at 100 V for 50 minutes. The gels were washed three times at room temperature for 30 minutes in distilled water containing 2.5% Triton X-100, and then twice at room temperature for 10 minutes in distilled water. After incubation in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, 10 mM CaCl₂, 0.02% Brij-35, 0.01% NaN₃) at 37°C for 20 hours, the gels were stained with 0.25% Coomassie brilliant blue R-250 (Sigma) for 1 hour and finally de-stained in 15% methanol/7.5% acetic acid. After de-staining, zones with enzymatic activity appeared as non-stained zones.

The quantitative analysis of the gelatinolytic enzyme was performed with a computer-assisted imaging densitometer system (Scion image, Scion Corporation). The 80 kDa MMP-9 and 66 kDa MMP-2 (active forms) and 92 kDa pro-MMP-9 and 72 kDa pro-MMP-2 were measured and compared with a standard protein marker (Amersham). Mean MMP activities

for each sample were derived from duplicate experiments.

Hydroxyproline assay

The ECM content was measured as a major protein component in the plasma by hydroxyproline assay. The hydroxyproline content in the plasma was modified from Reddy and Enwemeka (1996). Briefly, duplicate sets containing 50 µl sample plasma, 20 µl 10 N NaOH and 30 µl water were hydrolyzed in screw-capped tubes in boiling water for 3 hours. A hydrolysate solution was taken and supplemented with 100 µl chloramine T solution. Samples were incubated at room temperature for 20 minutes. Then, 200 µl freshly prepared Ehrlich's reagent was added to the tubes and incubated in a 65°C water bath for 20 minutes. Samples were transferred to a 96-well plate and read for absorbance at 540 nm. Hydroxyproline (Sigma) was used as the standard.

Histology

The periductal fibrosis was assessed by Masson's trichrome staining of 5 µm-thick paraffin sections.

Statistical analysis

Data were presented as means ± SE. The *t*-test was used to compare between groups, while ANOVA was used to compare three or more groups. Statistical analyses were done using SPSS version 11. A *p*-value of < 0.05 was required for statistical significance.

RESULTS

Histopathological changes in hamster opisthorchiasis and effect of praziquantel treatment

The profile of fibrotic changes in the liver was evaluated using Masson's trichrome (Fig 1). An increase in periductal fibrosis was observed around bile ducts in a time-dependent manner, as inflammatory cells decreased (Fig 1a). The periductal fibrosis in the chronic infection group (4 months) was thicker than that of the acute infection group (21 days). After praziquantel treatment, the thickness of periductal fibrosis

in the acute infection group tended to decrease. However, periductal fibrosis in the chronic infection group tended to persist from 1 month to 6 months after drug treatment (Fig 1b).

Hydroxyproline content in the plasma of hamsters infected with *O. viverrini* and effect of praziquantel treatment

The plasma hydroxyproline level increased in a time-dependent manner in *O. viverrini*-infected hamsters (Fig 2). A significant increase in plasma hydroxyproline content was observed between day 51 and 300, whereas the content was unchanged in the uninfected control groups. The plasma hydroxyproline level was associated with the thickening of periductal fibrosis in the *O. viverrini*-infected groups.

After praziquantel treatment, the hydroxyproline content gradually decreased compared with the infected control groups whether acute or chronic (Fig 3). The hydroxyproline content the chronic infection group was significantly higher than that of the acute infection group (*p* < 0.05). The profile of the hydroxyproline level in the acute infection group was also significantly lower and closer to a normal level than that of the chronic infection group (*p* < 0.01).

MMP-2 and MMP-9 activities in hamster opisthorchiasis: effect of praziquantel

The time-course study of MMP-2 and MMP-9 activities in the plasma of hamsters infected with *O. viverrini* is shown in Fig 4. Gelatin zymography revealed that the active MMP-2 (66 kDa) and MMP-9 (80 kDa) were observed in the plasma at all time points (Fig 4a). In the *O. viverrini*-infected control group, the activity of MMP-9 (80 kDa) reached its highest intensity on day 51 then decreased until day 300. Active 80-kDa MMP-9 was stable from day 210 to day 300, although pro-MMP-9 (92 kDa) was also prominent. MMP-2 (66 kDa) activity reached its highest intensity on day 111 then was stable until day 300 (Fig 4a). In addition, the intensity of pro-MMP-2 (72 kDa) was unchanged compared to the normal control group. The significant increase in MMP-9 (80 kDa) activity was observed on day 21 and 51 compared with normal hamsters (*p* < 0.05 and *p* < 0.01, respectively) (Fig 4b).

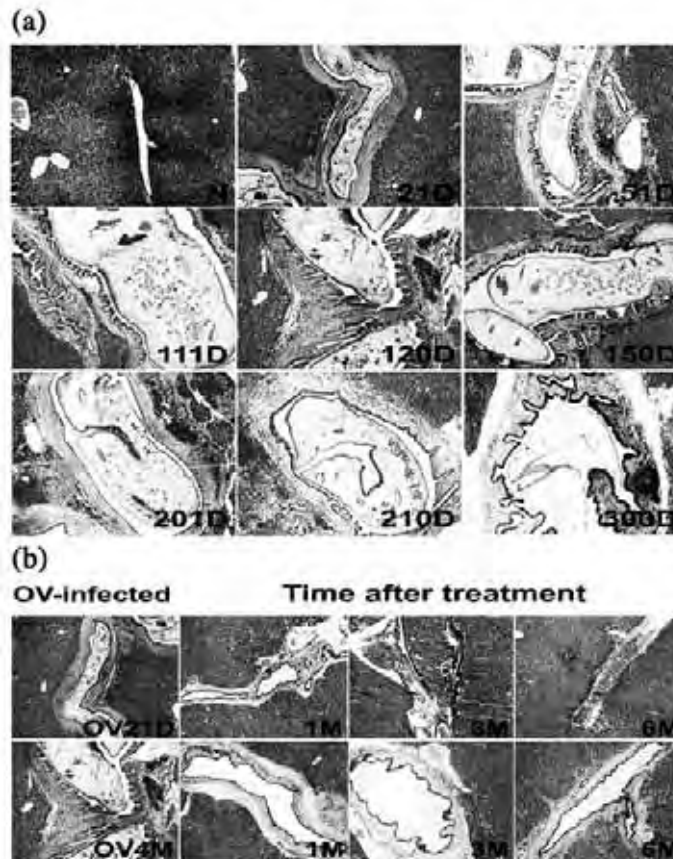


Fig 1- Histopathological features of hamster opisthorchiasis and effect of praziquantel treatment during acute (OV 21 days) and chronic (OV 4 months) infection. The periductal fibrosis was stained with Masson's trichrome. The dilatations of bile ducts and the thickness of periductal fibrosis increase with the duration of *O. viverrini* infection (a). After praziquantel treatment, the thickening of the periductal fibrosis in the 21-day infection groups tends to N = normal hamster, D = day, M = month decrease compared with those of the 4-month infection groups (b). The magnification is 40x.

MMP-2 and MMP-9 activities in groups treated with praziquantel after acute *O. viverrini* infection are shown in Fig 5. The activity of MMP-9 (80 kDa) decreased after praziquantel treatment from 1 month to 6 months, whereas the activity of MMP-2 (66 kDa) increased to 3 months then decreased after reaching 6 months (Fig 5b). By contrast, the intensity of pro-MMP-9 (92 kDa) was highest at 6 months after treatment. In addition, pro-MMP-2 (72 kDa) was stable after praziquantel treatment (Fig 5a).

MMP-2 and MMP-9 activities in groups with praziquantel treatment after chronic *O. viverrini* infection are shown in Fig 6. The activities of MMP-2 (66 kDa) and MMP-9 (80 kDa) decreased after praziquantel treatment, although MMP-2

activity was slightly increased at 1 month. The significant increase in MMP-9 (80 kDa) activity was observed at 1 and 3 months compared with normal hamsters (Fig 6b). By contrast, the intensity of pro-MMP-9 (92 kDa) was highest at 6 months after drug treatment. In addition, pro-MMP-2 (72 kDa) was stable from 1 to 6 months after praziquantel treatment (Fig 6a).

DISCUSSION

We first demonstrated MMP-2 and MMP-9 activities in *O. viverrini*-infected hamsters and then showed their relationship with respect to liver fibrosis resorption after praziquantel treatment. Praziquantel decreased the activities

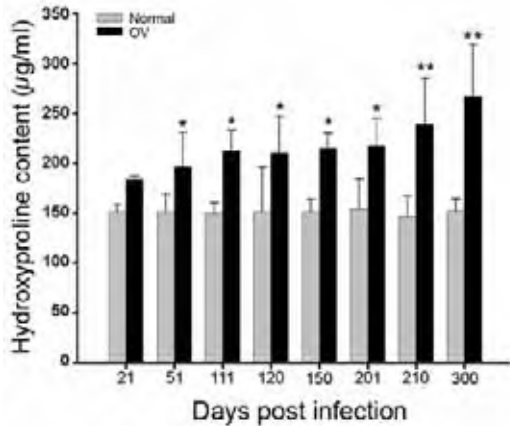


Fig 2- Hydroxyproline content in the plasma of *O. viverrini*-infected hamster compared with normal hamsters. The hydroxyproline level in the plasma increased in a time-dependent manner. A significant difference is observed on day 51 and throughout the study compared with the normal control groups. The *t*-test is used to compare between *O. viverrini*-infected and normal hamsters. Data are presented as mean \pm SE of five animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

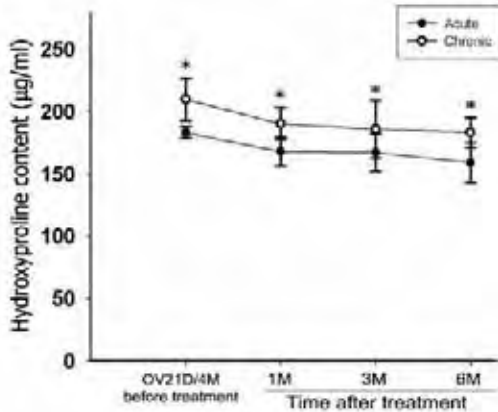


Fig 3- Effect of praziquantel treatment on hydroxyproline content in the plasma of hamsters infected with *O. viverrini* for 21 days and 4 months. After praziquantel treatment of the 21 days (acute) and 4 months (chronic), hydroxyproline levels tend to decrease from 1 to 6 months. The hydroxyproline content after drug treatment for the chronic groups is significantly higher than that of the acute groups ($p < 0.05$). The profile of hydroxyproline after drug treatment for the chronic groups is also significantly higher than that of the acute groups ($p < 0.01$). The *t*-test is used to compare between the acute and chronic groups at each time point and a one way ANOVA for comparisons of hydroxyproline profiles. The data are presented as means \pm SE of five animals. * $p < 0.05$.

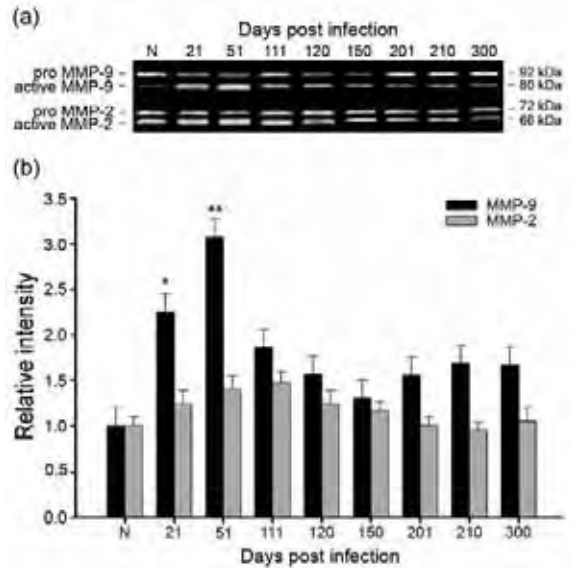


Fig 4- The profiles of MMP-2 and MMP-9 activities in the plasma of hamster opisthorchiasis. MMP-2 and MMP-9 activities were analyzed by gelatin zymography. MMP-9 (92 kDa and 80 kDa) and MMP-2 (72 kDa and 66 kDa) appear at all time points (a). The intensity of 80 kDa MMP-9 and 66 kDa MMP-2 (active form) reaches its maximum on day 51 and 111, respectively (b). The intensity of 72 kDa pro-MMP-2 is unchanged, whereas 92 kDa pro-MMP-9 gradually increases on day 51 then decreases until day 150. Afterward, its activity gradually increases and reaches a plateau (a). The relative intensity of the 80 kDa MMP-9 in the infected groups is significantly higher on day 21 and 51 post-infection compared with the normal control hamster (b). The *t*-test is used to compare between the infected and normal hamsters. Data are presented as mean \pm SE of five animals. N = normal control hamster, * $p < 0.05$ and ** $p < 0.01$.

of both MMP-2 and MMP-9 during the acute and chronic phases of *O. viverrini* infection. We hypothesized that MMP-2 and MMP-9 might play roles in the pathogenesis of opisthorchiasis through inflammation, and that praziquantel treatment decreases tissue resorption of periductal fibrosis.

Our hypothesis was apparently supported by evidence of MMP-9 and MMP-2 activity being at their highest on days 51 to 111 post-infection, respectively. Moreover, the inflammatory cells

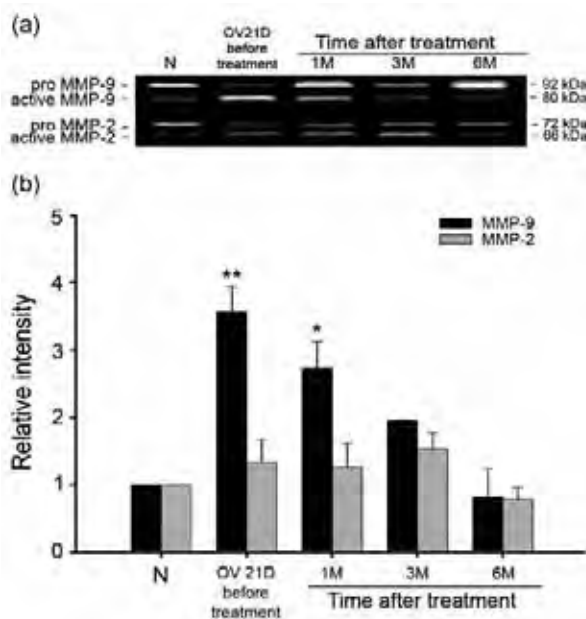


Fig 5- Effect of praziquantel treatment on MMP-2 and MMP-9 activities in plasma of *O. viverrini*-infected hamsters for 21 days. MMP-2 and MMP-9 activities were analyzed by gelatin zymography. The intensity of 80 kDa MMP-9 and 66 kDa MMP-2 (active form) gradually decreases from 1 to 6 months after drug treatment, whereas the intensity of 92 kDa pro-MMP-9 gradually increases and reaches its maximum at 6 months after drug treatment (a). The relative intensity of 80 kDa MMP-9 is significantly higher on day 21 post-infection and 1 month after praziquantel treatment compared with normal control hamsters (b). The *t*-test is used for a comparison between infected and normal hamsters (N). Data are presented as means \pm SE of five animals. * = $p < 0.05$ and ** = $p < 0.01$.

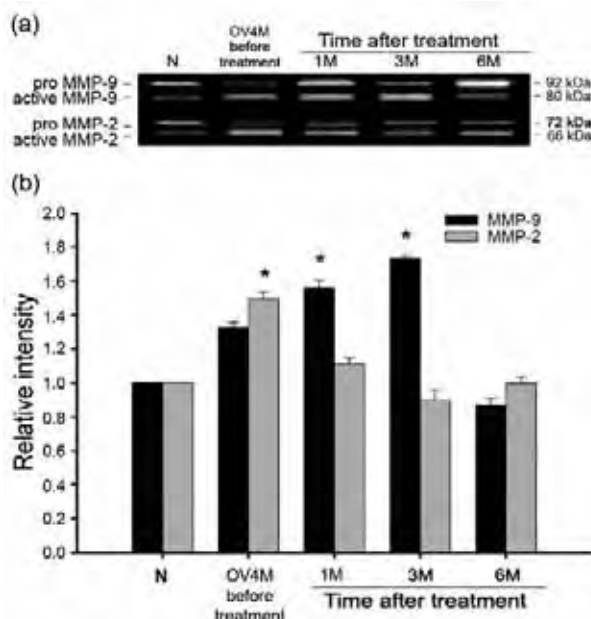


Fig 6- Effect of praziquantel treatment on MMP-2 and MMP-9 activities of *O. viverrini*-infected hamsters for 4 months. MMP-2 and MMP-9 activities were analyzed by gelatin zymography. The intensity of 80 kDa MMP-9 and 66 kDa MMP-2 (active form) gradually decreases, whereas 92 kDa pro-MMP-9 gradually increases (a). The relative intensities of 66 kDa MMP-2 and 80 kDa MMP-9 are significantly higher on 4 months post-infection and 1 and 3 months after praziquantel treatment, respectively, compared with normal control hamsters (b). The *t*-test is used to compare between infected and normal hamsters. Data are presented as means \pm SE of five animals. N = normal control hamster, * = $p < 0.05$.

were prominent in the same period. By contrast, pro-MMP-9 showed the most prominent intensity during chronic infection that was associated with the accumulation of periductal fibrosis, supported by the finding of hydroxyproline persistence in the plasma in the chronic phase and a correlation with periductal fibrosis.

Perhaps MMPs activities are based on a balance between pro-enzyme activation and inhibition by tissue inhibitors of MMPs (TIMPs) (Gomez *et al*, 1997). TIMPs promote the progression of periductal fibrosis through inhibition of matrix degradation (Iredale, 1997). Our study may elucidate a possible mechanism

for the accumulation of periductal fibrosis during chronic infection, as previously proposed by Pinlaor *et al* (2004). Therefore, MMP-2 and MMP-9 may play roles in tissue remodeling triggered by infection with *O. viverrini* and could therefore serve as markers of inflammation-associated opisthorchiasis rather than the fibrosis stage of chronic infection.

Praziquantel decreased the MMP-2 and MMP-9 activities and decreased collagen content in the liver after drug administration. Our results showed that the MMP-2 and MMP-9 activities gradually decreased after praziquantel treatment. Similarly, the hydroxyproline level tended to

decrease and was associated with the periductal fibrosis. Thus, we cannot exclude the activity of other MMPs in the resorption of periductal fibrosis triggered by *O. viverrini* infection.

After praziquantel treatment, the profile of MMP-9 activity in the acute phase (21-day infection) was higher than that of the chronic phase (4-month infection). This result is supported by the profile of the hydroxyproline level of the acute phase group, which was lower than that of the chronic phase group. Periductal fibrosis, as demonstrated by Masson trichrome, also helps to explain the hydroxyproline level.

Our results suggest that in chronic infection, MMPs may show a slow resorption of periductal fibrous tissue after drug treatment. Mice infected with *Schistosoma mansoni* showed a slow resorption of liver fibrous tissue following praziquantel treatment (Singh *et al*, 2004). The irreversible fibrosis may predispose to relative risk of primary sclerosing cholangitis, leading to CCA development. This hypothesis is supported by a previous study showing that irreversible fibrosis was not affected by drug treatment (Mairiang *et al*, 1993).

Early treatment (starting at 21 days post-infection) has yielded better resorption than treatment at 4 months post-infection. Our results are similar to those of rabbits infected with *Clonorchis sinensis* that showed that liver changes caused by acute clonorchiasis in the first two weeks were reversible if treated while chronic biliary epithelial changes were irreversible (Lee *et al*, 1987). Thus, to prevent the risk of opisthorchiasis-associated CCA development, early treatment should be recommended to minimize the remaining periductal fibrosis in opisthorchiasis.

Based on our results, MMP-2 and MMP-9 appear to be associated with the inflammation-mediated opisthorchiasis, and that praziquantel treatment, at the early stage, may enhance tissue resorption of periductal fibrosis. Other MMPs and TIMPs may play important roles in the tissue resorption of periductal fibrosis triggered by *O. viverrini* infection. Therefore, plasma MMP-2 and MMP-9 could be useful markers of opisthorchiasis and be used for the evaluation of the efficacy of praziquantel treatment.

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