EFFECT OF CURCUMIN ON THE INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) AND ANTIOXIDANT ENZYME EXPRESSION IN HAMSTERS INFECTED WITH *OPISTHORCHIS VIVERRINI*

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Abstract. Opisthorchiasis, caused by infection with the liver fluke *Opisthorchis viverrini*, is one of the major risk factors for cholangiocarcinoma (CCA). The imbalance between free radicals and antioxidant defense mechanisms causes oxidative and nitrative stress, leading to pathophysiological changes characteristic of the disease. A chemopreventive agent, such as curcumin, used to prevent inflammation may decrease the severity of the disease. The expression of inducible nitric oxide synthase (iNOS) and antioxidant enzymes were investigated in the livers of *O. viverrini*-infected hamsters with and without curcumin treatment using RT-PCR analysis. These results suggest that curcumin suppresses the expression of iNOS, whereas it enhances the expression of the antioxidant enzyme genes SOD1, CAT and GPx resulting in the inhibition of nitrative stress. Therefore, curcumin may be used as a chemopreventive agent to reduce the severity of opisthorchiasis as well as to prevent the development of CCA.

INTRODUCTION

Chronic inflammation is implicated in the pathogenesis and carcinogenesis of certain types of human cancers, including Opisthorchiasis viverrini-associated cholangiocarcinoma (CCA) (IARC, 1994; Ohshima and Bartsch, 1994; Coussens and Werb, 2002). Opisthorchiasis is caused by *O. viverrini* infection, the primary risk factor for cholangiocarcinoma (CCA) (IARC, 1994). The highest prevalence of *O. viverrini* infection and CCA in the world has been reported in northeast Thailand. Although praziquantel, an effective drug for opisthorchiasis, is widely used (Pungpak *et al*, 1998), the incidence of CCA is still a major public health problem in this region. This may be due to reinfection, which frequently occurs after praziquantel treatment in endemic areas because of the local diet of raw freshwater fish, the intermediate host (Saowakontha et al, 1993).

Intervention using a chemopreventive agent may reduce the severity of opisthorchiasis. At present, chemopreventive agents such as curcumin, a yellow pigment of the turmeric plant, has demonstrated properties involved in the suppression of tumor promotion through the inhibition of inflammation and antioxidant formation (Duvoix *et al*, 2005). Curcumin can also increase both bile secretion and production (Deters *et al*, 2000). This compound, a common spice in Thailand, may be used as an agent of choice for the reduction of pathogenesis characteristic of opisthorchiasis. Our experiment was designed to study the effect of curcumin on the pathogenesis of opisthorchiasis in an animal model that focused on the expression of inducible nitric oxide synthase (iNOS) and antioxidant enzymes-such as Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), catalase (CAT) and glutathione peroxidase (GPx) using semi-quantitative RT-PCR analysis. The anticipated outcome of this research may provide insight into a new therapeutic approach for opisthorchiasis as well as a strategy for reducing the risk of developing CCA.

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MATERIALS AND METHODS

Parasites

Opisthorchis viverrini metacercariae were isolated from naturally infected fish by pepsin digestion (Pinlaor *et al*, 2004). Briefly, cyprinoid fish were minced and digested in artificial pepsin-

HCl solution, sedimented several times in normal saline solution. Finally, the metacercariae of *O. viverrini* were collected under a dissecting microscope. Only viable cysts were used to infect the animals.

Curcumin was purchased from Sigma, and was mixed with the normal pellets to a concentration of 0.25% curcumin.

Animal and experimental design

Random bred male Syrian hamsters (obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University), 6-8 weeks of age and 90-150 g body weight, were used in the study. The hamsters were divided into four groups (5 animals/group): Group I: the normal hamster control group fed normal pellets; Group II: the normal hamster fed curcumin-supplemented pellets; Group III: the hamsters infected with 50 *O. viverrini* metacercariae and fed normal pellets; and, Group IV: the hamsters infected with 50 *O. viverrini* metacercariae and fed curcumin-supplemented pellets. All of the hamsters were kept at room temperature in the animal house and fed their designated diet with water given *ad libitum*.

On days 7, 14, 21, 30, 60, and 90, hamsters from Groups I and III were sacrificed, while hamsters in the curcumin-treated group were sacrificed on days 30 and 90. Samples of blood and liver (at the hilar region) were collected from each sacrificed animal and used for this study. The study was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

Histopathological study

A histopathological study was performed using hematoxylin and eosin staining of paraffin sections (Sripa and Kaewkes, 2000). Briefly, the liver tissues from the hilar region were fixed in 10% buffered formalin overnight then processed conventionally. The sections were then cut in 6-μm slices and stained with hematoxylin and eosin.

RT-PCR for the expression of iNOS and antioxidant enzyme genes in the liver of *O. viverrini*-infected hamsters with and without curcumin treatment

Primer design for semi-quantitative RT-PCR and sequence analysis. The iNOS primer was designed from GenBank (Accession No. AJ863052), *Mesocricetus auratus* partial mRNA for iNOS, iNOS, sense CAG CTT GGA GTT CAC CCA GT, and iNOS antisense CCA CTC GTA TTT GGG ATG CT (169 bp). The gene-specific primers of antioxidant genes (SOD1, SOD2, CAT, and GPx) were designed based on the published sequence of *Mus musculus* and *Rattus norvegicus* mRNA.

The deduced nucleotide sequences were aligned using the Clusta W software (http://www.

Table 1
Sequences of primers used and expected size of amplified fragments.

Genes	GenBank Accession numbers	Sequence 5'→ 3' Upper line: forward primer Bottom line: reverse primer	PCR product size (bp)
SOD1	NM_017050.1	F5' CGGATGAAGAGAGGCATGTT 3' R5' CACCTTTGCCCAAGTCATCT 3'	165
SOD2	NM_017051.2	F5' CCGAGGAGAAGTACCACGAG 3' R5' GCTTGATAGCCTCCAGCAAC 3'	174
GPx	NM_030826.2	F5' GGTTCGAGCCCAACTTTACA 3' R5' CGGGGACCAAATGATGTACT 3'	152
CAT	NM_012520.1	F5' TTGACAGAGAGCGGATTCCT 3' R5' AGCTGAGCCTGACTCTCCAG 3'	179
MG3PDH	M32599	F5' GGCATTGTGGAAGGGCTCAT 3' R5' GACACATTGGGGGTAGGAACAC3'	218

ebi.ac.uk/clustaw/). Searches for homologies were done using the Fasta3 software (http://www.ebi.ac.uk/fasta3/). General "homology" searches were done using the Blast software from the NCBI home page (BLASTn, http://www.ncbi.nlm.nih.gov./BLAST/).

The expected sizes of the amplified fragments are presented in Table 1. In addition, the primer pairs for endogenous controls (glyceraldehyde-3-phosphate dehydrogenase, G3PDH) was designed based on the published sequence (Boonmars *et al*, 2005).

RNA preparation. Total RNA was isolated from each of the hamster livers using TRIZOL® reagent (Invitrogen), according to the manufacturer's instructions. Approximately 150 mg of hamster liver was dissected and immediately dipped into the reagent. The isolated RNA was further processed (Boonmars *et al*, 2005). Briefly, the total RNA was treated with DNase (5 units of RQ1 RNase-Free DNase, Promega), and 100 units of ribonuclease inhibitor (Recombinant RNase Inhibitor, Promega) in a buffer (containing, 400 mM Tris-HCl, 100 mM NaCl, 60 mM $MgCl_2$, and 20 mM dithiothreitol, pH 7.5).

Total RNA was then extracted with phenol/chloroform, precipitated with ethanol, and dissolved in RNase-free water (100 µl). The extracted RNA was reverse-transcribed into cDNA using Oligo (dT) 15 primers (Promega). The PCR reaction mixture comprised 2 µl of reverse transcription products (1:10 diluted), 2 µl of 10x PCR buffer, 2 µl of deoxynucleoside triphosphate (2.5 mM dNTP, each), 2 µl of primer pairs (10 pmol), 0.08 µl of *Ex Taq* polymerase (5 U/µl, Takara, Shuzo, Japan), and distilled water was added to a final volume of 20 µl. PCR analysis was performed using a Thermocycler (GeneAmp®, PCR system 9700), in which all the target genes remained in the exponential phase for 30-40 cycles.

PCR reactions were conducted: 94°C for 3 minutes; and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds for SOD1, SOD2, GPx; whereas, iNOS and CAT were performed for 40 cycles. The resulting PCR products were visualized on 2% agarose gel. Gel images were digitally captured, and the fragment intensity was determined using the Scion image (Herolab, Wiesloch). All reactions for the standard control and the experimental sample were performed in triplicate. The amount of iNOS and antioxidant genes (*viz*, SOD1, SOD2, and GPx) were calculated relative to the expression of the control gene, G3PDH. Fragment identity was confirmed after cloning into home-made T-vector followed by sequencing using the respective gene specific primers with the Cy5 label primer (Applied Biosystems) on MegaBACE™ 1000 DNA analysis System (Pharmacia).

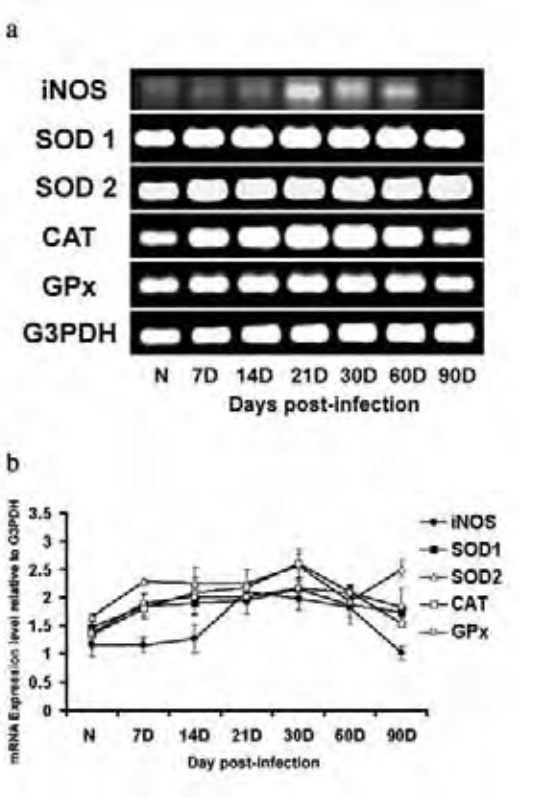

Fig 1- RT-PCR analysis of the expression of iNOS and antioxidant enzyme genes in the liver of *O. viverrini*-infected hamsters. The representative gel image for iNOS, antioxidant enzyme genes and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression in *O. viverrini*-infected hamsters and in normal hamsters is shown in 2% agarose gel (a). The expression level is represented by the ratio of the intensity of the sample gene relative to G3PDH (b) of three hamsters. The data comprise means ± SD for three hamsters per group. N = normal hamsters, D = day.

RESULTS

The mRNA expression profiles of iNOS, SOD1, SOD2, CAT, and GPx

The time-interval profiles of mRNA expression of iNOS, SOD1, SOD2, CAT and GPx in the liver of *O. viverrini*-infected hamsters are shown in Fig 1. The specific band of G3PDH had unchanged intensity throughout the time-course study (Fig 1a). The specific bands of iNOS, SOD1, SOD2, CAT, and GPx were also observed throughout the time-course study. Semi-quantitative RT-PCR analysis showed that the respective profiles of SOD1, CAT, and GPx gene expression in the liver of *O. viverrini*-infected hamsters gradually increased on day 7, reached a peak on day 30, and tended to decrease by day 90 (Fig 1b). In addition, iNOS expression reached its peak on day 21; but the expression profile of SOD2 reached two peaks, one on day 30 and another on day 90.

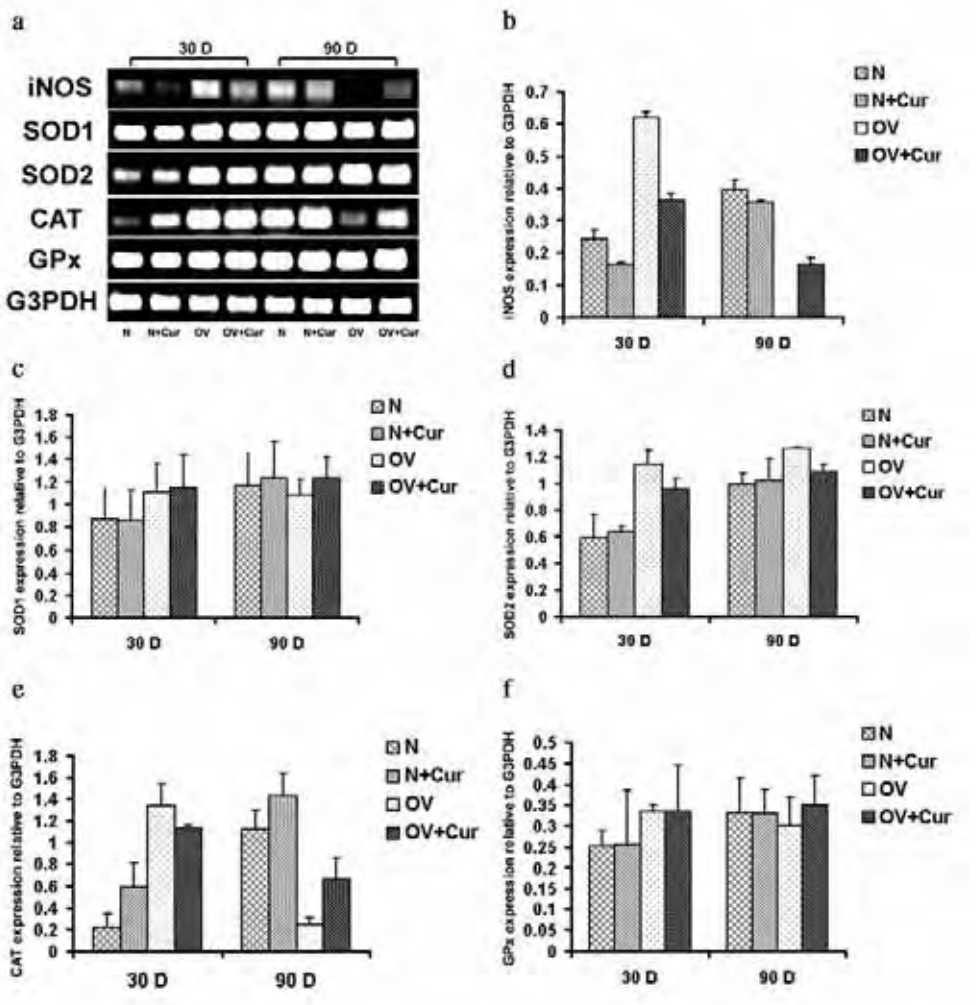

Fig 2- RT-PCR analysis of the effect of curcumin on the mRNA expression of iNOS and antioxidant enzyme genes in the liver of *O. viverrini*-infected hamsters. The representative gel image for iNOS, antioxidant enzyme genes and G3PDH expression in *O. viverrini*-infected hamsters supplemented with curcumin are shown in 2% agarose gel (a). The expression level is represented by the ratio of the intensity of the sample gene relative to G3PDH (b) in three hamsters. N = normal hamsters treated with normal pellets, N+Cur = normal hamsters treated with curcumin, OV = *O. viverrini*, OV+Cur = *O. viverrini*-infected hamsters and administration with curcumin.

Effect of curcumin on the mRNA expression of iNOS, SOD1, SOD2, CAT and GPx

The effect of curcumin on the mRNA expression of iNOS, SOD1, SOD2, CAT, and GPx in the liver of *O. viverrini*-infected hamsters is shown in Fig 2. The intensity of specific band of G3PDH on days 30 and 90 was the same (Fig 2a). The specific bands for iNOS, SOD1, SOD2, CAT, and GPx were also observed on days 30 and 90. The expression of iNOS was lower (0.37-fold) in the curcumin-treated group than the 0.62-fold in the *O. viverrini*-infected group on day 30 (Fig 2b). The expression of SOD2 was lower (1.00-fold on day 30 and 1.08-fold on day 90) in the curcumin-treated group than in the *O. viverrini*-infected group (1.20-fold on day 30 and 1.26-fold on day 90) (Fig 2d). By contrast, SOD1 expression was higher (1.28-fold) in the curcumin-treated group than the 1.13-fold in the *O. viverrini*-infected group on day 90 (Fig 2c). CAT expression was higher (0.66-fold) in the curcumin-treated group than the *O. viverrini*-infected group (0.24-fold) on day 90 (Fig 2e). GPx expression was higher (0.35-fold) in the curcumin-treated group than the in the *O. viverrini*-infected group (0.30-fold) on day 90 (Fig 2f).

Effect of curcumin on the histopathological changes

The effects of curcumin on the histopathological changes in the liver of *O. viverrini*-infected hamsters are shown in Fig 3. Curcumin did not significantly reduced the accumulation of inflammatory cells around the bile ducts on day 30 compared with the untreated group (Fig 3). The thickening of the walls of the gallbladder and extrahepatic bile ducts was less on day 90 in the curcumin-treated group when compared to the untreated group (Fig 4).

DISCUSSION

The present study is the first report on the effect of curcumin on the pathological changes caused by opisthorchiasis through the iNOS and antioxidant enzyme gene expression. Curcumin seems to reduce the accumulation of inflammatory cells around the bile ducts and to decrease the thickness of the gallbladder wall and extrahepatic bile duct in experimental opisthorchiasis. The mRNA expression of iNOS and SOD2 was decreased by curcumin treatment, whereas the antioxidant enzyme genes, such as SOD1, CAT, and GPx, increased the level of mRNA expression after curcumin treatment. We therefore hypothesize that curcumin suppresses the expression of iNOS, whereas it enhances the expression of antioxidant enzyme genes (SOD1, CAT, and GPx) resulting in the inhibition of nitrative stress and reduces the severity of opisthorchiasis.

In *O. viverrini*-infected animals, increases in

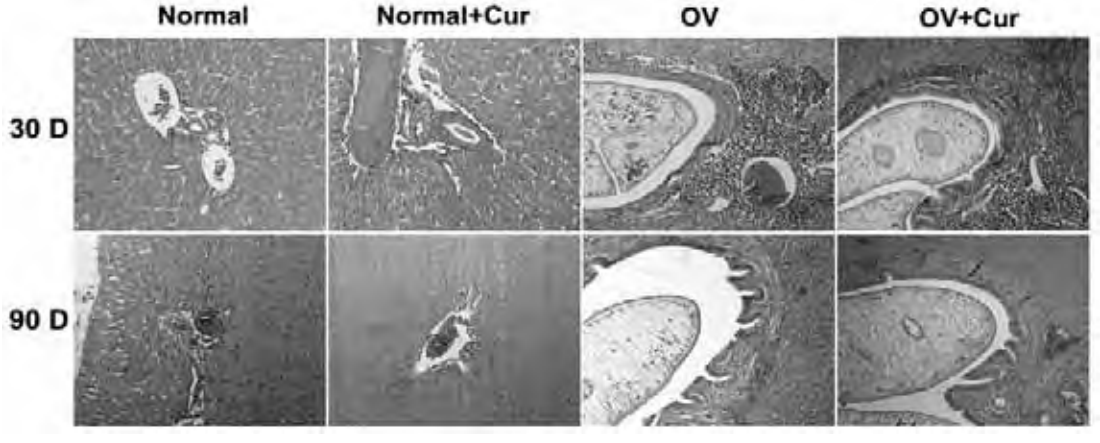

Fig 3- Histopathological studies of the livers of untreated and curcumin-treated hamsters. The histopathological changes in *O. viverrini*-infected hamster livers stained using hematoxylin-eosin. Original magnification is 40x. D = day, Cur = curcumin, OV = *O. viverrini*, OV+Cur = *O. viverrini*-infected hamsters and administration with curcumin.

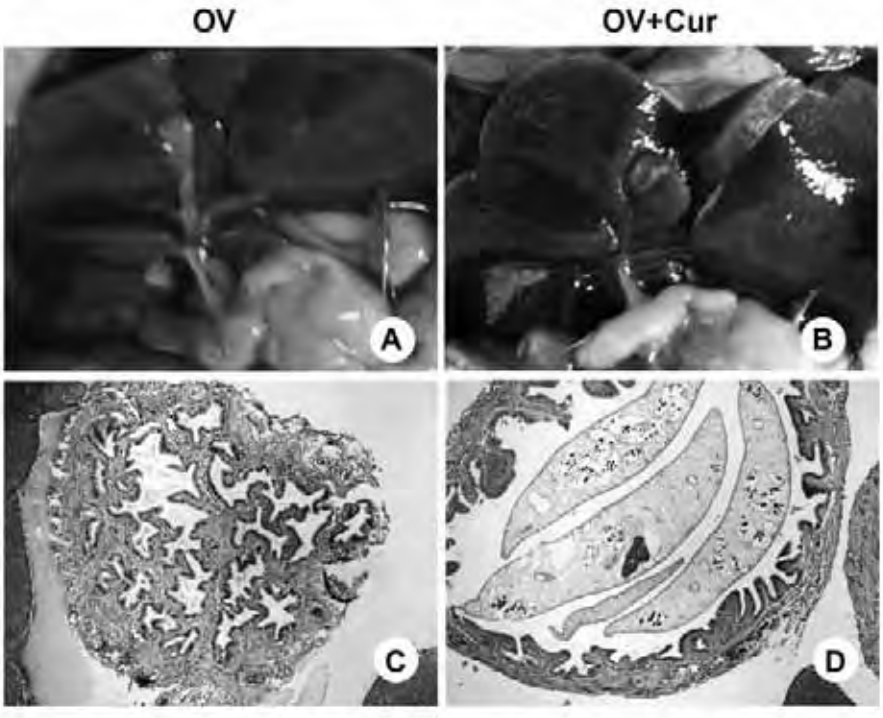

Fig 4- Effect of curcumin on the extrahepatic bile ducts and the gallbladder of untreated and curcumin-treated hamsters. Presented here are the gross appearance (in the first row) and histopathological changes (in the second row) of the extrahepatic bile duct and the gallbladder of *O. viverrini*-infected hamsters (A, C) and curcumin-treated *O. viverrini*-infected hamsters (B, D) on day 90. Original magnification is 40x. OV = *O. viverrini*, OV+Cur = *O. viverrini*-infected hamsters and administration with curcumin.

the mRNA expression of iNOS and the antioxidant enzyme genes (*ie*, SOD1, SOD2, CAT, and GPx) were associated with the accumulation of inflammatory cells around the bile duct, as with previous reports (Bhamarapravati *et al*, 1978; Sripa and Kaewkes, 2000; Pinlaor *et al*, 2004). The expression of iNOS is directly related to nitric oxide (NO) production. Over-expression of iNOS leads to increased damage to NO-mediated biomolecules (*ie*, oxidative and nitrative DNA damage) (Pinlaor *et al*, 2003). Similarly, iNOS-associated NO production has been reported in mice infected with *Schistosoma mansoni* (Brunet *et al*, 1999) and *Trichinella spiralis* (Garside *et al*, 2000). Excess expression of iNOS leading to a large amount of NO production is therefore involved in the pathology of various parasitic infections (Brunet *et al*, 1999; Garside *et al*, 2000). iNOS-mediated DNA damage through the oxidation and nitration reaction in hamster infected with *O. viverrini* may be a risk factor for the development of CCA (Pinlaor *et al*, 2004). Therefore, iNOS expression may increase nitrative stress and result in the pathological changes characteristic in opisthorchiasis.

The host defense against cell injury via oxidative and nitrative damage is through the induction of antioxidant enzyme expression. Our results showed that the expression of SOD1, CAT, and GPx gradually increased, reached their peaks on day 30, and then tended to decrease by day 90. This was perhaps because the first line of antioxidant enzyme defense to the overproduction of superoxide anion (O_2•-) is the SODs (such as SOD1 and SOD2) which catalyze the O_2•- into hydrogen peroxide (H_2O_2) and O_2 to protect biomolecules from injury (Kim *et al*, 2005). GPx and CAT mRNA expression is the second order of induction to catalyze H_2O_2 into a less toxic substance, that is, H_2O and O_2. Changes in the antioxidant enzyme gene expression in the liver and in the phospholipid structure of

the cell membrane is accompanied by raising the liver damage in rats infected with *Fasciola hepatica* (Kolodziejczyk *et al*, 2005). However, over-expression of SOD1 and SOD2 also partially protected liver injury from the increasing reactive oxygen species production. Therefore, the increase in the expression of antioxidant enzymes may act as a useful mechanism in the host to prevent oxidative and nitrative stress triggered by *O. viverrini* infection.

The decrease in mRNA expression on day 30 for iNOS, and on days 30 and 90 for SOD2, in the present experiment suggests that curcumin might play a role in inhibiting the constitutive NF-kappaB and IKK activities (Aggarwal *et al*, 2005; Surh *et al*, 2001). The inhibiting effect of curcumin on iNOS and SOD2 may be the result of the suppression of nuclear factor kappaB translocation (Karin and Greten, 2005). Curcumin can also reduce the iNOS mRNA in the livers of lipopolysaccharide-injected mice (Mabbott *et al*, 1998) and inhibit NO generation in mice infected with *Leishmania* (Chan *et al*, 2005). By contrast, curcumin can enhance the expression of SOD1, CAT, and GPx. The increase in the expression of antioxidant enzyme genes may have a beneficial effect as a host defense mechanism by reducing the severity of opisthorchiasis. This idea is supported by the observation that curcumin increased the mRNA expression of SOD1, CAT and GPx on day 90. The inflammatory cells around the bile ducts seemed to be decreased on day 30, and the extrahepatic bile duct had reduced thickening wall on day 90. Similarly, curcumin increases activities of SODs and CAT enzymes thus preventing oxidative damage in the cataracts of rats (Padmaja and Raju, 2004). Curcumin also reportedly reduces the severity of diseases in mice infected with malaria (Reddy *et al*, 2005) and coccidian infection in chickens (Allen *et al*, 1998).

In conclusion, the results of our experiment indicate that curcumin may (1) reduce the pathogenesis of opisthorchiasis through the suppression of iNOS and SOD2, and (2) enhance the expression of SOD1, CAT, and GPx thereby preventing oxidative and nitrative stress. Our results suggest that curcumin may have a beneficial effect on opisthorchiasis and be a useful chemopreventive treatment agent for prevention of *O. viverrini*-associated CCA development.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from (1) the Graduate School, (2) the Faculty of Medicine Research Fund, (3) the Khon Kaen University Research Fund, and (4) the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. We thank Mr Bryan Roderick Hamman for assistance with the English-language presentation of the manuscript.

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