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Research Article

Curcumin reduces oxidative and nitrative DNA damage through balancing of oxidant–antioxidant status in hamsters infected with *Opisthorchis viverrini*

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Opisthorchis viverrini (OV) infection is endemic in northeastern Thailand. We have previously reported that OV infection induces oxidative and nitrative DNA damage *via* chronic inflammation, which contributes to the disease and cholangiocarcinogenesis. Here, we examined the effect of curcumin, an antioxidant, on pathogenesis in OV-infected hamsters. DNA lesions were detected by double immunofluorescence and the hepatic expression of oxidant-generating and antioxidant genes was assessed by quantitative RT-PCR analysis. Dietary 1.0% curcumin significantly decreased OV-induced accumulation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an oxidative DNA lesion, and 8-nitroguanine, a nitrative DNA lesion, in the nucleus of bile duct epithelial and inflammatory cells. Expression of oxidant-generating genes (inducible nitric oxide synthase; iNOS, its nuclear transcriptional factor, NF-κB, and cyclooxygenase-2), and plasma levels of nitrate, malondialdehyde, and alanine aminotransferase, were also decreased in curcumin-treated group. In contrast, curcumin increased the mRNA expression of antioxidant enzymes (Mn-superoxide dismutase and catalase), and ferric-reducing anti-oxidant power in the plasma. In conclusion, curcumin reduced oxidative and nitrative DNA damage by suppression of oxidant-generating genes and enhancement of antioxidant genes, leading to inhibition of oxidative and nitrative stress. Therefore, curcumin may be used as a chemopreventive agent to reduce the severity of OV–associated diseases and the risk of cholangiocarcinoma (CCA).

Keywords: Curcumin / 8-nitroguanine / *Opisthorchis viverrini* / 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) / Oxidant and antioxidant genes

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1 Introduction

Infection with liver fluke, *Opisthorchis viverrini* (OV), is endemic in Southeast Asia including Thailand, Lao PDR, Vietnam, and Cambodia [1]. Approximately 6 million people are estimated to be infected with the liver fluke in Thailand [2]. The highest prevalence of OV infection has been found in the Northeast Thailand and is associated with the high incidence rate of cholangiocarcinoma (CCA) [3]. Infection induces the hepatobiliary disease and is a relative risk factor for development of CCA [1], which is believed to be caused *via* chronic inflammation [3–5].

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Abbreviation: CCA, cholangiocarcinoma; **iNOS,** inducible nitric oxide synthase; **NO,** nitric oxide; $O_2^{\bullet-}$**,** superoxide anion radical; $ONOO^-$**,** peroxynitrite; **OV,** *Opisthorchis viverrini*; **8-oxodG,** 8-oxo-7,8-dihydro-2′-deoxyguanosine

Excess free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated from inflammatory and epithelial cells, and these cells therefore are considered to play a key role in several pathological conditions including carcinogenesis [5–8]. OV infection has been shown to mediate iNOS-dependent DNA damage in intrahepatic bile duct epithelium and inflammatory cells of animals [9, 10]. We have reported that inflammation caused by infection with OV induces the accumulation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an oxidative DNA lesion, and 8-nitroguanine, a mutagenic nitrative DNA lesion, which may contribute to related diseases and cholangiocarcinogenesis [10]. In addition, the sensitive balance between the oxidant and antioxidant forces in the body appears to be very crucial in determining health and longevity [11]. An imbalance of oxidant-generating and antioxidant genes or gene products may lead to oxidative and nitrative stress, contributing to the development of disease [12]. Therefore, intervention by chemopreventive agents may reduce DNA damage and prevent CCA.

Curcumin, the active ingredient in turmeric (*Curcuma longa*), has been proposed for the treatment of several diseases, including various cancers [13]. Curcumin has been shown to reduce inflammation and the generation of free radical and carcinogenesis, and it is inexpensive and has been found to be safe in human clinical trials [13, 14]. We have hypothesized that curcumin may reduce oxidative and nitrative DNA damage through inhibition of inflammation and balancing of oxidant-antioxidant mechanisms.

To clarify the role of curcumin in reducing oxidative and nitrative DNA damage caused by OV infection, accumulation of 8-oxodG, and 8-nitroguanine was assessed by double immunofluorescence. To evaluate the proliferation of bile duct epithelial cells, we examined expression of proliferating cell nuclear antigen (PCNA). Hepatic expression of oxidant-generating genes such as iNOS, its transcriptional factor NF-κB, cyclooxygenase-2 (COX-2), and antioxidant genes that encode Cu/Zn-superoxide dismutase (SOD1), Mn-SOD (SOD2), extracellular SOD (SOD3), catalase (CAT) and glutathione peroxidase (GPx), was assessed by quantitative reverse transcription (RT)-PCR analysis. Biochemical parameters including the plasma levels of nitrate (end products of nitric oxide (NO)), the plasma malondialdehyde (MDA, an oxidative biomarker), the ferric-reducing anti-oxidant power (FRAP), and alanine aminotransferase (ALT, an index of liver injury) activity in the plasma was also analyzed. In addition, histopathological changes in the liver were studied by hematoxylin and eosin.

2 Materials and methods

2.1 Parasites

OV metacercariae were isolated from naturally infected fish by pepsin digestion as described previously [9]. Cyprinoid fish, obtained from the endemic area of Khon Kaen province, Thailand, were digested by artificial 0.25% pepsin-HCl. OV metacercariae were collected under a dissecting microscope and viable cysts were used to infect hamsters.

2.2 Experimental animals

The Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (Reference No.0514.1.12/436) approved this study. Four to six week-old male golden hamsters (*Mesocricetus auratus*) were housed under conventional conditions and fed a stock diet and given water *ad libitum*. In the OV-infected group, hamsters were infected with 50 metacercaria of OV by oral inoculation and treated with normal diet. In the curcumin-treated group, curcumin (Cayman, USA, purity 75%) was dissolved in corn oil (final concentration <1%) and mixed with the homogenized normal diet, subsequently was made in a pellet, dried and kept at room temperature and protected from sunlight. Dietary curcumin was prepared once a week.

2.2.1 Determination of optimal dose of dietary curcumin for protective effect on liver injury of OV-infected hamsters

Because of limited data for optimal doses of curcumin in hamster models, we examined a preventive effect of various doses of curcumin supplemented in normal diet on liver injury in OV-infected hamsters. Fifty male hamsters were divided into two groups consisting of hamsters with and without OV infection. Each group was divided into five subgroups (five animals each), which were given diet supplemented with 0, 0.1, 0.25, 0.5, and 1% w/w curcumin. After 30 days post-treatment, they were sacrificed and plasma was collected to measure ALT activity to evaluate the liver injury. ALT activity was determined by using automate analyzer (Synchron Clinical System, CX^{TM}4, Beckman, USA). The most effective dose of curcumin (1%) was used in subsequent experiments.

2.2.2 Effect of curcumin on DNA damage, oxidative and nitrative stress and histological changes in OV-infected hamsters

We investigated the effect of curcumin on DNA damage, oxidative, and nitrative stress and histopathological changes in OV-infected hamsters. Eighty animals were used to investigate the effect of curcumin on OV-infected hamsters for 14, 21, 30, and 90 days, and 20 animals were used for each time point. Animals at each time point included four groups; normal hamsters fed with normal diet (N), normal hamsters and fed with 1% w/w curcumin-supplemented diet (N + Cur), OV-infected hamsters fed with normal diet (OV), and OV-infected hamsters fed with 1% curcumin-supplemented diet (OV + Cur). Five animals were used in

each experimental group and sacrificed at time points described above.

2.3 Specimen collections

After the period of OV infection and curcumin treatment, hamsters were anaesthetized with ether, and then liver tissues were collected. The liver sections of 0.5 cm in diameter (approximately 150 mg) were taken from the hilar region and adjacent areas including the secondary order bile duct lumens, where the worms are usually found, and were used for either total RNA isolation or histopathological study. For total RNA isolation, the livers were immediately treated with TRIZOL™ reagent for RNA isolation and then stored at –80°C until use. For histopathological and immunohistochemical study, livers were fixed in 10% buffered formalin. In addition, the blood was collected from the heart into a tube containing EDTA. After centrifugation at 5000 g for 5 min, plasma was collected and stored at –80°C until analysis.

2.4 Immunohistochemical study

Because our previous data showed the peak of NO production and 8-nitroguanine formation in the liver of hamsters infected with OV for 30 days, we determined the most effective dose of curcumin on DNA lesions at this time point by using immunohistochemical study [9, 10]. Co-localization of 8-nitroguanine and 8-oxodG formation in the liver was assessed by double immunofluorescence labeling study as described previously [9]. Briefly, paraffin sections (5 μm thickness) were deparaffinized in xylene and rehydrated in descending gradations of ethanol. To enhance the immunostaining, the sections were placed in citrate buffer (pH 6.0) and autoclaved at 120°C for 10 min for antigen unmasking. In addition, to demonstrate 8-nitroguanine formation in DNA more clearly, tissue sections were pretreated with 10 μg/mL RNase at 37°C for 1 h according to the method described previously [15]. We produced highly sensitive and specific anti-8-nitroguanine rabbit polyclonal antibody as described previously [9] and used as primary antibody at a concentration of 2 μg/mL, and a mouse monoclonal anti-8-oxodG antibody (2 μg/mL, Japan Institute for the Control of Aging, Fukuroi, Japan) overnight at room temperature. Next, the sections were incubated for 3 h with an Alexa 594-labeled goat antibody against rabbit IgG and an Alexa 488-labeled goat antibody against mouse IgG (each, 1:400, Molecular Probes Inc., Eugene, Oregon, USA). The stained sections were examined using a fluorescence microscope.

In addition, the localization of iNOS and PCNA in the hamster liver was also assessed by indirect immunofluorescence labeling study as described previously at the corresponding time point with DNA lesions [9]. Paraffin sections were incubated with the primary antibodies [rabbit polyclonal anti-iNOS antibody (1:400, Abcam, Cambridge Science Park, Cambridge, UK), and mouse monoclonal anti-PCNA antibody (1:100, Santa Cruz Biotechnology, Inc., USA)] overnight at room temperature. Samples were then incubated with secondary antibodies against mouse or rabbit primary antibodies as described above.

The histopathological change was assessed by hematoxylin eosin staining in paraffin sections.

2.5 Isolation of mRNA and cDNA production

Approximately 150 mg of the hamster liver from the hilar region including the secondary order bile duct lumen (where worms inhabit) was harvested for total RNA isolation using TRIZOL™ (Invitrogen, USA), according to the manufacturer's instructions. Total RNA was treated with 5 units of DNase (Promega, USA) and 119 units of Ribonuclease Inhibitor (Promega, USA). Total RNA (3 μg) was reverse-transcribed into cDNA using Oligo(dT) 15 primers (Promega, USA) following the protocol for transcription by M-MLV reverse transcriptase (Promega, USA).

The oligonucleotide-specific primer pairs for identification of hamster-related genes were designed based on GenBank accession nos. XM342346.2 for NF-κB, DQ355357.1 for iNOS, AF345331.1 for COX-2, NM017050.1 for SOD1, NM017051.2 for SOD2, AB212861.1 for SOD3, NM012520.1 for CAT, NM030826.2 for GPx, and DQ403054.1 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primer sequences are as follows: for NF-κB, forward (GCTTTGCAAACCTGGGAATA), reverse (CAAGGTCAGAATGCACCAGA); for iNOS, forward: (CAGAACAGAGGGCTCAAAGG), reverse (TCTGCAGGATGTCTTGAACG); for COX-2, forward: (CCATGGGTGTGAAAGGAAAT), reverse (GAAGTGCTGGGCAAAGAATG); for SOD1, forward (CGGATGAAGAGAGGCATGTT), reverse (CACCTTTGCCCAAGTCATCT); for SOD2, forward (CCGAGGAGAAGTACCACGAG), reverse (GCTTGATAGCCTCCAGCAAC); for SOD3, forward (GGCAGCCCTTGGCTCTGT), reverse (AAAGCTGGACTCCGCAGGAT), for CAT, forward (TTGACAGAGAGCGGATTCCT), reverse (AGCTGAGCCTGACTCTCCAG), for GPx, forward (GGTTCG AGCCCAACTTTACA), reverse (CGGGGACCAAATGAT GTACT), for GAPDH, forward (AGAAGACTGTGGATGGCCCC), reverse (TGACCTTGCCCACAGCCTT). GAPDH was used as an endogenous control [16] and all primer sets had a calculated annealing temperature of 55°C.

2.6 Cloning of PCR product

To confirm the PCR fragment, RT-PCR was performed using a Thermocycler (GeneAmp®, PCR system 9700). Fragment identity was confirmed after cloning into homemade T-vector, followed by sequencing using the respective

Table 1. Sequences of amplified fragments and their percent identities to homologous rat, mouse and human sequences

Genes	Sequence (5′ → 3′)	cDNA fragment size	% Nucleotide (% amino acid) identities[a)]		
			Rat	Mouse	Human
iNOS	CAGAACAGAGGGCTCAAAGGAGGCCGCAT-GACCTTGGTGTTTGGGTGCCGGCACCCAGAGGAGGAC-CACCTCTATCGGGAAGAGATGCAGGAGATGGCCCACAAGG-GAGTGCTGCACCAGGTGCATACAGCCTACTCCAGGCTGC-CAGGCAAGCCCAAGGTCTACGTTCAAGACATCCTGCAGA	184	92 (90)	91 (85)	91 (90)
NF-κB	GCTTTGCAAACCTGGGAATACTTCATGTAACTAAG-CAAAAGGTATTTGCAACACTGGAGGCCCGGATGACA-GAGGCGTGTATACGGGGCTACAATCCTGGACTTCTGGTG-CATTCTGACCTTG	123	91 (95)	93 (95)	-(92)
COX-2	CCATGGGTGTGAAAGGAAATAAGGAGCTTCCTGATTC-GAAAGAAGTTCTGGAAAAAGTTCTTCTCAGGAGAAAGTT-CATCCCTGATCCCCAAGGCACGAATATGATGTTTG-CATTCTTTGCCCAGCACTTC	131	93 (97)	92 (95)	86 (81)
SOD1	CGGATGAAGAGAGGCATGTTGGGGACCTGGGCAACGT-GACTGCTGGGAAGGATGGTGTGGCCACTGTGTCCATTGAA-GACCCTGTGATCTCTCTCTCAGGAGAGCACTCCAT-CATTGGCCGAACGATGGTGGTCCATGAGAAGCAAGAT-GACTTGGGCAAAGGTG	165	91 (94)	92 (96)	86 (87)
SOD2	CCGAGGAGAAGTACCACGAGGCCCTGGCCAAGGGA-GATGTTACGACTCAGATTGCTCTTCAACCTGCCCT-GAAAGTTCAATGGTGGGGGACATATCAATCACAG-CATTTTCTGGACAAACCTGAGCCCTAATGGTGGTGGA-GAGCCCAAAGGAGAGTTGCTGGAGGCTATCAAGC	175	94 (97)	93 (94)	89 (100)
SOD3	GTGCACCAGATAACTCCCTATGATG-GAGGTTCCCTCCGTTCTTGACACTCCACTT-TAAGGGCCCTCTGTGTCCCGATAACCACACAAGCCCTTAG-CATCCCCTTTGAAACAGTCTTTGAGTCTGTTTGCTTCC	133	97	–	–
CAT	TTGACAGAGAGCGGATTCCTGAGAGAGTGGTGCATG-CAAAGGGAGCAGGTGCCTTTGGATACTTTGAGGTCACT-CACGATATTACCAGGTACTGTAAGGCAAAGGTGTTTGAGCA-CATTGGAAAGAGGACCCCCATTGCCGTTCGATTCTCCA-CAGTCGCTGGAGAGTCAGGCTCAGCT	179	93 (98)	93 (98)	84 (94)
GPx	GGTTCGAGCCCAACTTTACATTGTTCGAAAAGTGCGAGGT-CAATGGTGAGAAGGCTCACCCGCTCTTTACCTTCCTGCGG-GAGTCCTTGCCAGCGCCCAGTGACGACCCGACTGCGCT-CATGACCGACCCCAAGTACATCATTTGGTCCCCG	152	94 (96)	92 (94)	81 (84)
GAPDH	AGAAGACTGTGGATGGCCCCTCCGGGAAGCTGTGGCGT-GATGGCCGTGGGGCTGCCCAGAACATCATCCCTGCATC-CACTGGTGCTGCCAAGGCTGTGGGCAAGGTCA	108	97 (100)	98 (100)	93 (97)

a) The sequence identities are shown for the portion of the sequence corresponding to the hamster cDNA reported here. The amino acid sequence is deduced from the nucleotide sequence. No data have been provided as shown in "-". The GenBank accession numbers used in the sequence comparison of rat, mouse and human were as follows: iNOS, NM012611.2 (CAA54208.1), NM010927.1 (AAH62378.1), AF049656 (AAC83553.1); NF-κB, XM001075876.1 (EDL82271.1), NM008689.2 (EDL12139.1), – (EAX06134.1); COX-2, AF233596.1 (AAN52933.1), NM011198.3 (AAA39918.1), AY462100.1 (AAA57317.1); SOD1, BC082800.1 (NP058746.1), BC086886.1 (NP035564.1), EF151142.1 (AAP36703.1); SOD2, NM017051.2 (CAA30928.1), NM008160.5 (AAH86649.1), BC007865.2 (CAA68491.1); SOD3, BC061861.1; CAT, BC081853.1 (EDL79667.1), BC013447.1 (AAH13447.1), BC110398.1 (AAK29181.1); GPx, NM030826.3 (CAA30928.1), NM008160.5 (AAH86649.1), BC007865.2 (CAA68491.1); GAPDH, NW047470.1(ABD77186.1), XR033853.1 (XP001473673.1), and NT009759 (88014556). The GenBank accession numbers are present as nucleotide (protein) database for rat, mouse and human, respectively.

gene specific primers with the Cy5-labeled primer (Applied Biosystems, USA) on MegaBACE™ 1000 DNA analysis System (Pharmacia, USA). The DNA sequences were analyzed using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit). BLAST was used to search the nucleotide and protein database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov./BLASTn or BLASTX). Sequences of amplified fragments and the percent identities for the alignments to homologous rat, mouse, and human sequences are shown in Table 1.

2.7 Quantitative RT-PCR analysis

Relative mRNA expression was determined by ABI7500 thermal cycler using a SYBR Green assay. Three replicates were performed for each experiment. A PCR reaction (20 μL) contained 5 μL of single stranded cDNA, PCR buffer, 0.25 mM of each dNTP, 5 pmol of forward and reverse primers, 0.5X SYBR Green and 1 U of Hot start *Taq* polymerase (MBI Fermantas, St. Leon-Rot, Germany). The PCR cycling conditions were 95°C for 10 min, then 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min. After PCR, a dissociation curve was constructed in the range of 60–99°C according to the dissociation protocol of the instrument.

Equivalent PCR efficiency was confirmed for each primer set from the slope of the standard curve of serial two-fold dilutions. Dissociation curves always indicated the formation of a single PCR product. All data were analyzed using the Rotor Gene 5 software (Corbett, Australia) with a cycle threshold (Ct) in the linear range of amplification and then processed by the $2^{-\Delta\Delta Ct}$ method [17]. Relative gene expression was calibrated using expression of GAPDH gene and reported as fold change over background levels in the normal control sample.

2.8 Analysis of biochemical parameters

The amount of NO production was determined as nitrate in the plasma by the vanadium-based simple spectrophotometric method using the Griess reaction [18] with minor modifications. The assay was performed in a standard flat-bottomed, 96-well, polystyrene microtiter plate. The nitrate concentration in the biological samples was measured after catalyzed reduction to nitrite using vanadium (III) chloride (VCl_3). Plasma (100 μL) was deproteinized to reduce turbidity with 200 μL of cold absolute methanol to diethyl ether (3:1, v/v) for 30 min at –80°C. The samples were centrifuged at 12 000 g for 10 min and the supernatant was analyzed. After 100 μL of the supernatant or standard nitrate was added to a well, 100 μL of VCl_3 was added, followed by immediate addition of 100 μL of the Griess reagent (premixed 50 μL of 2% sulfanilamide in 5% HCl and 50 μL of 0.1% of *N*-(1-naphthyl) ethylenediamine dihydrochloride). The contents were vigorously mixed and the plate was incubated for 20 min at 37°C and read absorbance at 540 nm.

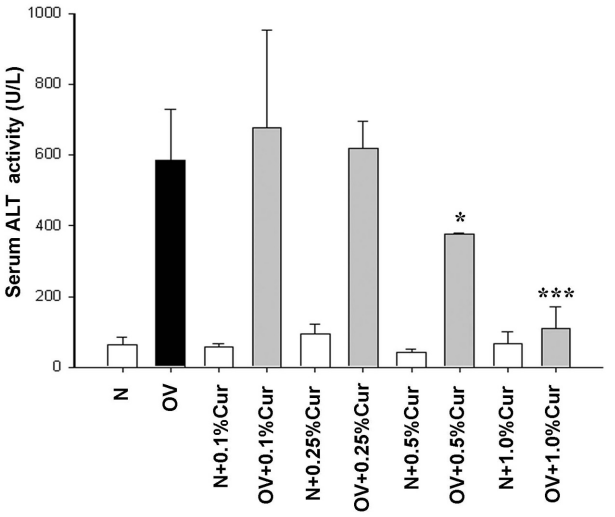

Figure 1. Optimal protective dose of curcumin on ALT activity in the plasma. Fifty male hamsters were divided into two groups consisting of hamsters with and without OV infection. Each group was divided into five subgroups (five animals each), which were given diet supplemented with 0, 0.1, 0.25, 0.5, and 1% w/w curcumin for 30 days. ALT activity in the plasma was measured by using automate analyzer. Data present as mean ± SD of five animals and experiment was performed in duplicate. Statistical significance was analyzed using Student's *t*-test to compare OV-infected hamsters with and without OV treated with curcumin treatment. $^{*}P < 0.05$, and $^{***}P < 0.001$.

TBA-reactive substances (TBARS) reaction equivalent to MDA was measured as described previously [19]. Briefly, 200 μL of plasma and 8.1% SDS, 1.5 mL of 0.5 M HCl, 1.5 mL of 20 mM TBA, 50 μL of 7.2% BHT in 95% ethanol, and 550 μL of deionized water (DI) were added to a screw-capped test tube. The sample was stirred and heated by boiling in a heater (90°C) for 15 min. After cooling for 10 min in an ice box, the chromogen was extracted by adding 1 mL of DI and 5 mL of *n*-butanol-pyridine solution. The sample was mixed thoroughly and centrifuged at 3000 g for 15 min. TBARS adduct with MDA, TBAR-MDA was determined against reagent blank by a spectrofluorometry (GEMINI, XPS, Molecular Devices, CA, USA) with 520 nm excitation and 550 nm emission, using 1,1,3,3-tetramethoxypropane as the standard.

FRAP, antioxidant power, in the plasma was measured by spectrophotometer [20]. One thousand μL of FRAP reagent [300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka Chemicals, Switzerland), and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$), ratio 10:1:1] was incubated with 30 μL of plasma or standard calibrator ($FeSO_4 \cdot 7H_2O$) for 4 min at 37°C. The reaction was stopped by adding sulfuric acid (0.1 M H_2SO_4 in 0.5 M Tris-HCl, pH 6.8). The change in absorbance at 620 nm between the final reading and the

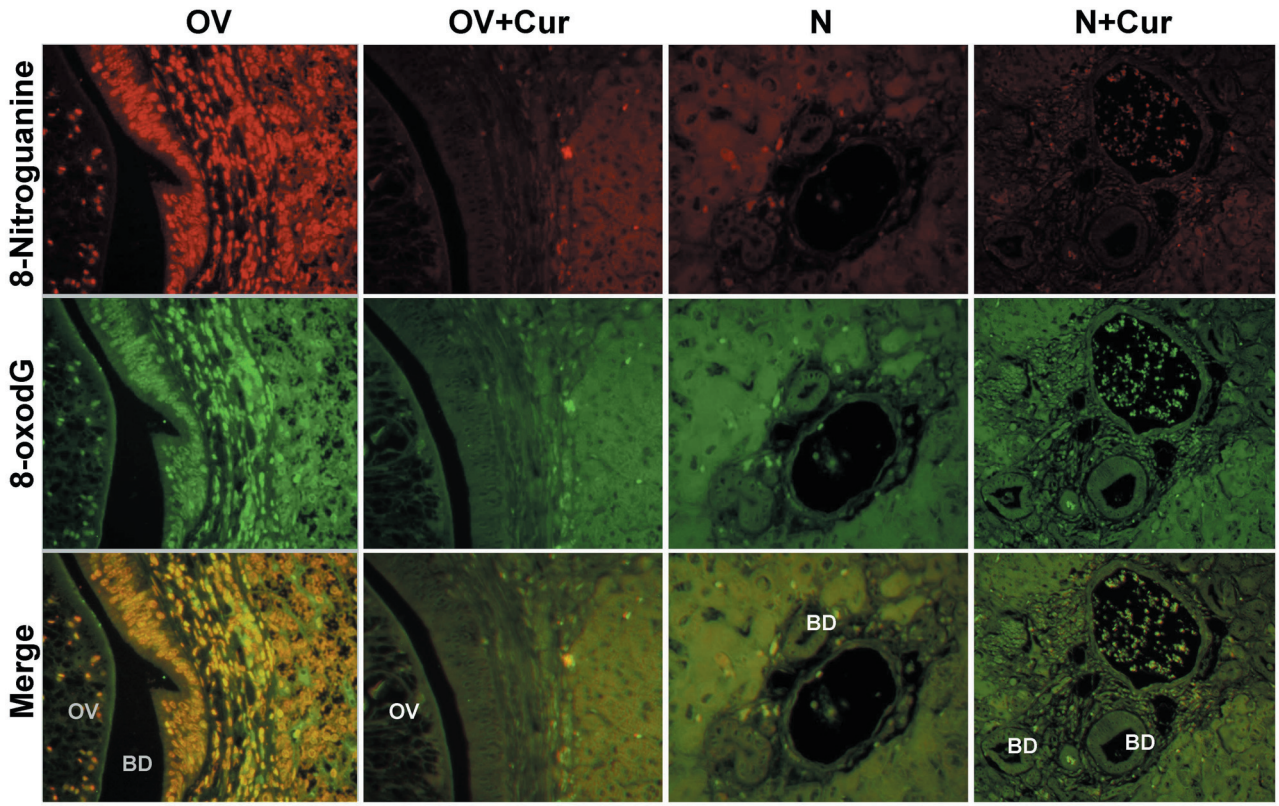

Figure 2. Protective effect of curcumin against DNA damage. Accumulation of 8-oxodG and 8-nitroguanine in the livers of hamsters with or without 1% curcumin treatment was assessed by double immunofluorescence technique as described in Materials and methods. Animals were divided into 4 groups as described in Materials and methods section: normal hamsters fed with normal diet (N), normal hamsters and fed with 1% w/w curcumin-supplemented diet (N + Cur), OV-infected hamsters fed with normal diet (OV), and OV-infected hamsters fed with 1% curcumin-supplemented diet (OV + Cur). These animals were sacrificed after OV-infection and/or curcumin treatment for 30 days. Positive immunoreactivity of 8-oxodG (green), 8-nitroguanine (red), and merge (yellow), is shown in the nucleus of bile duct epithelial cells and inflammatory cells in OV-infected hamsters and disappeared in curcumin-treated animals. The magnification was 400x. OV = *O. viverrini*, BD = bile duct.

reagent blank reading was calculated for each sample, using Fe^{II} as a standard.

2.9 Statistical analysis

Data were presented as means ± SD. The Student's *t*-test was used to compare between two groups and One-Way Analysis of Variance was used to test for differences among two or more independent groups. Statistical analyses were performed using SPSS version 11.5. Statistical significance in immunoreactivities was determined using the χ^2-test. *P* values less than 0.05 were considered statistically significant.

3 Results

3.1 Optimal dose of curcumin on the activity of the liver enzyme ALT

To determine the effective dose of curcumin that can protect against liver injury, plasma ALT activity was examined in various doses of dietary curcumin (final concentration; 0.1, 0.25, 0.5 and 1%) in the experimental animals at 1 month post-treatment (Fig. 1). OV infection significantly increased plasma ALT activity compared with normal control. In the curcumin-treated group, a significant decrease of ALT activity was observed at 0.5 and 1%. Notably, 1% curcumin decreased ALT activity to the normal level. In addition, ALT activity in normal hamster treated with curcumin was unchanged compared with normal control at any dose.

3.2 Inhibitory effect of curcumin on DNA damage

Since 1% dietary curcumin had the highest protective effect against liver injury on days 30 post-treatment, we assessed the effect of this dose of curcumin on the accumulation of 8-oxodG, and 8-nitroguanine in the hamster liver at the corresponding time point by double immunofluorescence technique (Fig. 2). In OV infection group, strong immunoreactivity of 8-oxodG and 8-nitroguanine was observed in the nucleus of bile duct epithelial cells and inflammatory cells.

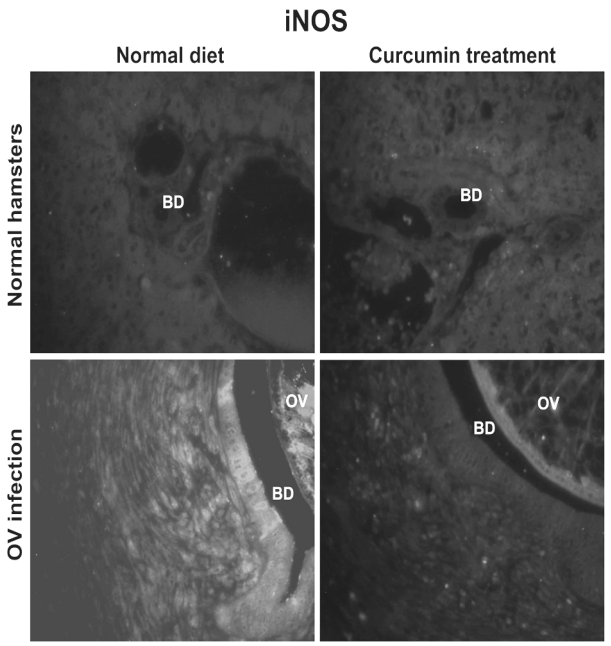

Figure 3. Protective effect of curcumin on iNOS expression in the liver of hamsters infected with OV. Expression of iNOS in the liver of hamsters infected with OV was assessed by indirect immunofluorescence technique as described in Materials and methods. A strong immunoreactivity of iNOS appears in the cytoplasm of bile duct epithelial cells and inflammatory cells in OV-infected hamsters and disappears in curcumin-treated animals. The experiment was performed as described in Figure 2 legend. The magnification was 400x. OV = *O. viverrini*, BD = bile duct.

In contrast, curcumin treatment significantly decreased the immunoreactivity of 8-oxodG and 8-nitroguanine in hamster liver. In addition, little or no immunoreactivity of these DNA lesions was observed in normal hamsters with or without curcumin treatment.

3.3 Inhibitory effect of curcumin on iNOS and PCNA expression

Figure 3 shows the expression of iNOS in the liver of hamsters infected with OV and the effect of curcumin treatment on days 30 post-treatment. OV induced strong immunoreactivity of iNOS in the cytoplasm of bile duct epithelium and inflammatory cells at the inflammatory area. In contrast, little immunoreactivity of iNOS was observed in OV-infected hamsters treated with curcumin. In addition, little or no immunoreactivity of iNOS was observed in the normal hamsters treated with 1% curcumin and in the normal control group.

Figure 4 shows the expression of PCNA, which acts as a response for cell proliferation during DNA synthesis and is involved in DNA repair, in the liver of hamsters infected with OV with and without curcumin treatment on days 30 post-treatment. OV induced strong immunoreactivity of

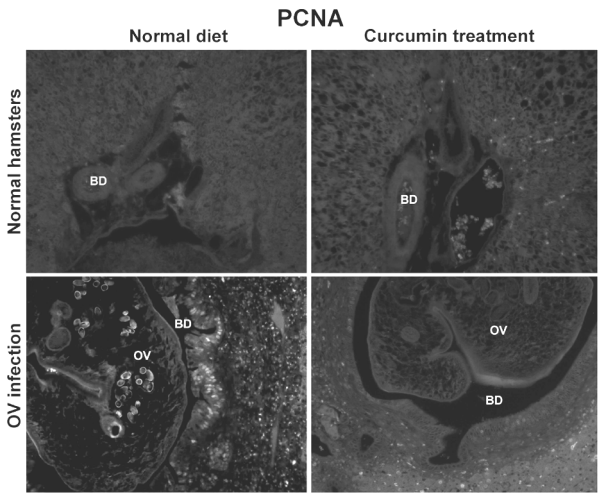

Figure 4. Protective effect of dietary curcumin on PCNA expression in the liver of hamster infected with OV. Expression of PCNA in the liver of hamsters infected with OV was assessed by indirect immunofluorescence technique as described in Materials and methods. Strong immunoreactivity of PCNA appears in the nucleus of bile duct epithelial cells and inflammatory cells in OV-infected hamsters and disappears in curcumin-treated animals. The experiment was performed as described in Fig. 2 legend. The magnification was 200x. OV = *O. viverrini*, BD = bile duct.

PCNA in the nucleus of bile duct epithelial cells and inflammatory cells at the site of inflammation. In contrast, a significant decrease in immunoreactivity of PCNA was observed after 1% dietary curcumin treatment. In addition, little or no immunoreactivity of PCNA was observed in the normal hamster with 1% curcumin treatment and in the normal control group.

3.4 Protective effect of curcumin on histopathological changes in the liver of hamsters infected with OV and on OV egg production

Figure 5 shows the effect of curcumin on histopathological changes in the liver of hamsters infected with OV. OV induced the dilatation of bile ducts and the thickness of periductal fibrosis increased with the duration of infection. The observed histopathological changes were similar to previously published data [21]. Interestingly, although the dilatation of bile ducts was increased time-dependently, the accumulation of inflammatory cells and the periductal fibrosis was reduced after 1% dietary curcumin treatment. In addition, curcumin had no effect on the normal hamster liver even in curcumin-treated or untreated groups.

To examine the effect of curcumin on worms laying eggs, two days before animals were sacrificed in OV and OV + Cur groups, feces were collected for parasite egg examination using the formalin ether concentration tech-

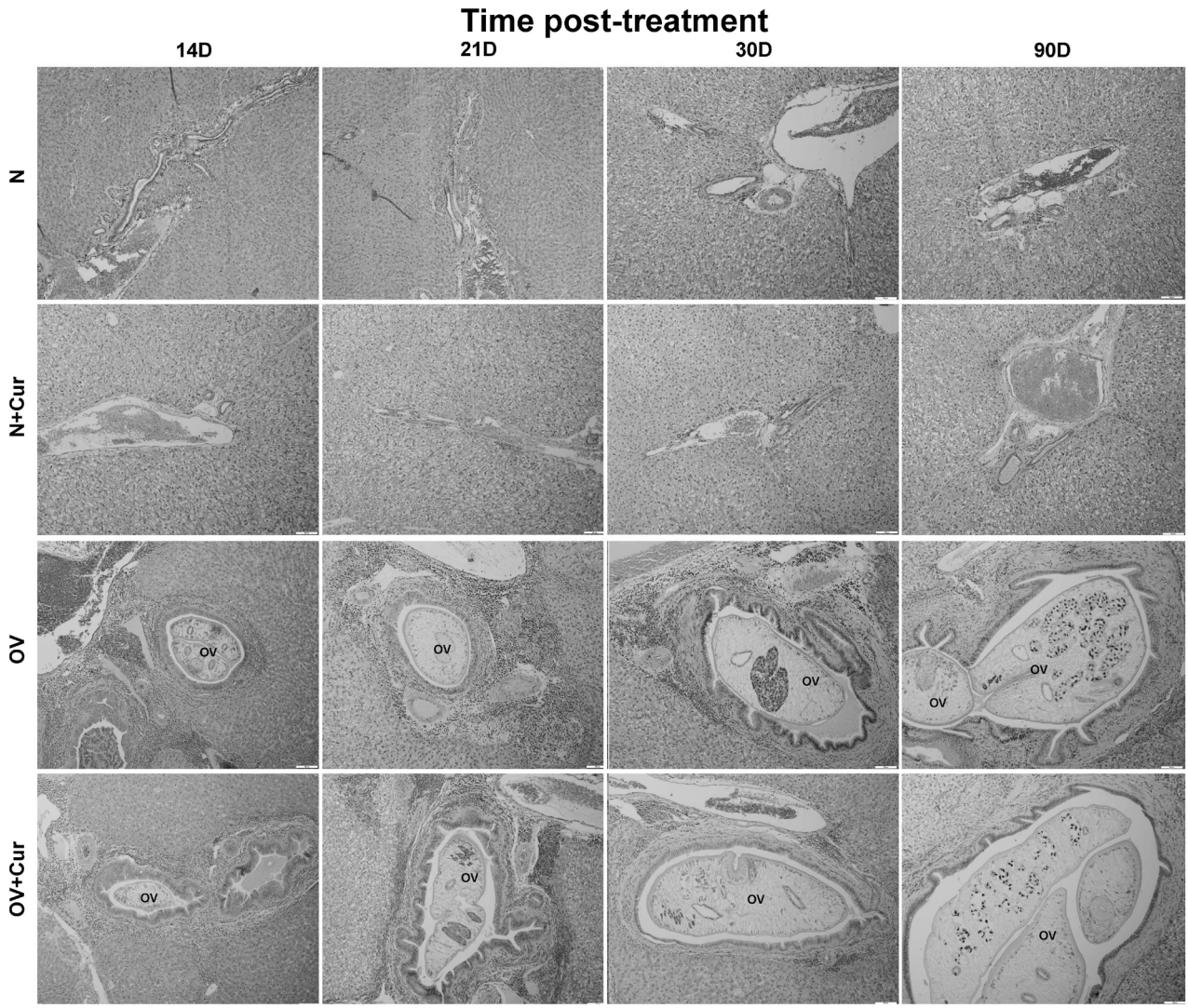

Figure 5. Protective effect of curcumin on histopathological changes in the liver of hamsters infected with OV. Histopathological changes in the liver of hamsters infected with OV and the protective effect of curcumin were performed by hematoxylin and eosin staining as described in Materials and methods. Animals were treated with OV and/or curcumin for 14, 21, 30, and 90 days, and a group of each time point was further divided into four subgroups; normal hamsters fed with normal diet (N), normal hamsters and fed with 1% w/w curcumin-supplemented diet (N + Cur), OV-infected hamsters fed with normal diet (OV), and OV-infected hamsters fed with 1% curcumin-supplemented diet (OV + Cur). The magnification was 100x. OV = *O. viverrini*.

nique. No significant difference in the number of OV eggs per gram of feces was found between these groups (data not shown).

3.5 Protective effect of curcumin on the expression of oxidant-generating genes in the liver of hamster infected with OV

On the basis of rodent genes, we studied oxidant-generating genes, such as iNOS, its transcription factor NF-κB and COX-2. Effect of curcumin on the mRNA expression profiles of oxidant genes in the liver of hamsters infected with OV are shown in Fig. 6. The nucleotide sequences of these genes were more similar (>91%) to rat and mouse, than humans. Genbank accession numbers are shown in Table 1.

After OV infection, mRNA expression of NF-κB, transcriptional regulator of various oxidant genes, and iNOS significantly increased on day 14 and reached a peak on day 21, and tended to decrease thereafter. Expression of COX-2 gene significantly increased on day 14–30 and decreased on day 90, but its level was still significantly higher than that in normal control group. Interestingly, 1% dietary curcumin significantly suppressed the expression of iNOS, NF-κB and COX-2 mRNA at different time points. A significant decrease in NF-κB was observed at day 21 and 90 post-treatment compared with hamsters without curcumin sup-

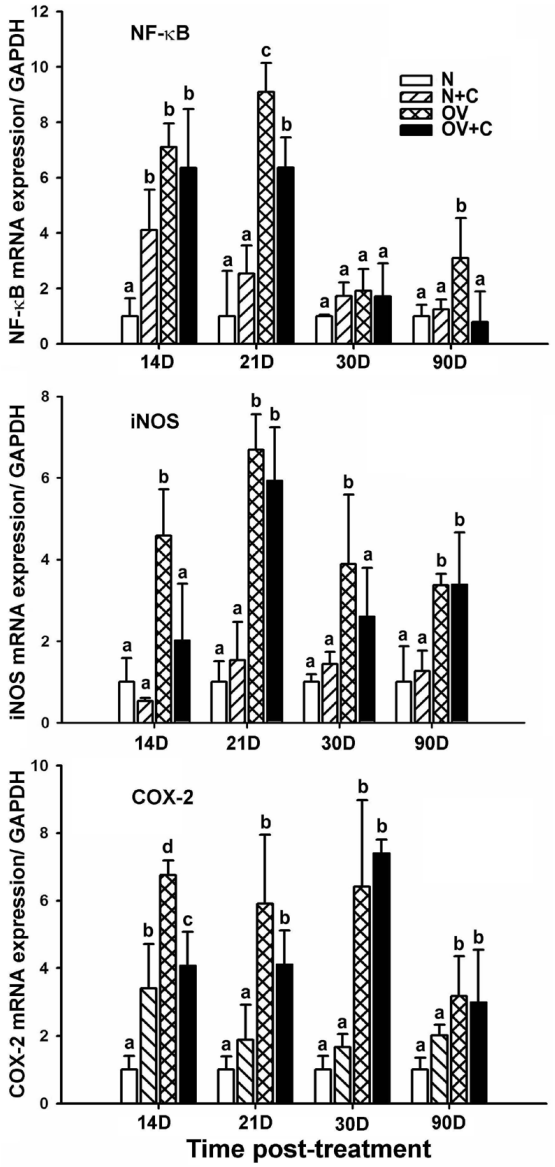

Figure 6. Protective effect of curcumin on the expression of oxidant-generating genes in the liver of hamsters infected with OV. Relative mRNA expression of oxidant-generating genes such as NF-κB, iNOS, and COX-2 to GAPDH was assessed by quantitative RT-PCR as described in Materials and methods. Animals were treated with OV and/or curcumin and divided into 4 groups as described in Fig. 5 legend: normal hamsters fed with normal diet (N), normal hamsters and fed with 1% w/w curcumin-supplemented diet (N + Cur), OV-infected hamsters fed with normal diet (OV), and OV-infected hamsters fed with 1% curcumin-supplemented diet (OV + Cur). Data are presented as the mean ± SD of 3 animals and triplicate set of experiment was performed. One way ANOVA was used to compare among these four groups at each time point. a, b, c, and d are the statistical significance among all groups ($P < 0.05$). Different alphabets in the same group mean statistically significant difference in the order d > c > b > a for each time point. D = day(s).

plement. Curcumin also significantly decreased the mRNA expression of iNOS on day 14 and 30, and COX-2 on day 14 and 21 post-treatment. In addition, in normal hamster with curcumin treatment, these genes transcripts were unchanged compared with normal control group, except NF-κB and COX-2 which increased at day 14 post-treatment.

3.6 Protective effect of curcumin on the expression of antioxidant genes in the liver of hamsters infected with OV

Effect of curcumin on the hepatic mRNA expression profile of gene encoding antioxidant enzymes such as SOD1, SOD2, SOD3, CAT, and GPx in the liver of hamsters infected with OV is shown in Fig. 7. OV infection increased the expression of SOD3 gene on day 14, and an increase in SOD2 and CAT expression was observed on day 21 post-infection. By day 30 post-infection, SOD2 was still increased, but CAT decreased thereafter. Expression of SOD1 gene slightly increased than that in normal control group. Interestingly, the increased expression of SOD2 and CAT genes was observed in OV-infected hamsters with curcumin treatment throughout the experimental period compared with those with OV infection alone. Curcumin also increased SOD3 expression on day 90 post-treatment. In contrast, curcumin had no effect on SOD1 mRNA expression. In addition, expression of GPx mRNA was unchanged in all experimental groups (data not shown).

3.7 Protective effect of curcumin on biochemical parameters in the plasma of hamsters infected with OV

Figure 8 shows the plasma levels of nitrate (Fig. 8A), MDA (Fig. 8B), ALT activity (Fig. 8C), and FRAP (Fig. 8D), and the effect of curcumin treatment. OV infection increased plasma nitrate, plasma MDA, and ALT activity, on days 14–90 post-infection. After infection, ALT gradually increased and reached a peak on day 21 and decreased thereafter, but its level was still higher than that normal control group. Interestingly, 1% dietary curcumin treatment significantly decreased plasma level of nitrate and ALT activity on days 14–30 post-treatment. Furthermore, curcumin significantly decreased MDA level throughout the time course (days 14–90). In addition, the plasma level of these biochemical parameters was unchanged in normal hamsters treated with curcumin compared with the normal control group.

OV infection also increased plasma level of ferric reducing antioxidant power (FRAP) in a similar pattern to ALT activity. In contrast to oxidative and nitrative stress marker and liver injury markers, plasma FRAP significantly increased by curcumin supplement on day 14–90 post-treatment. In addition, the plasma FRAP level was unchanged in

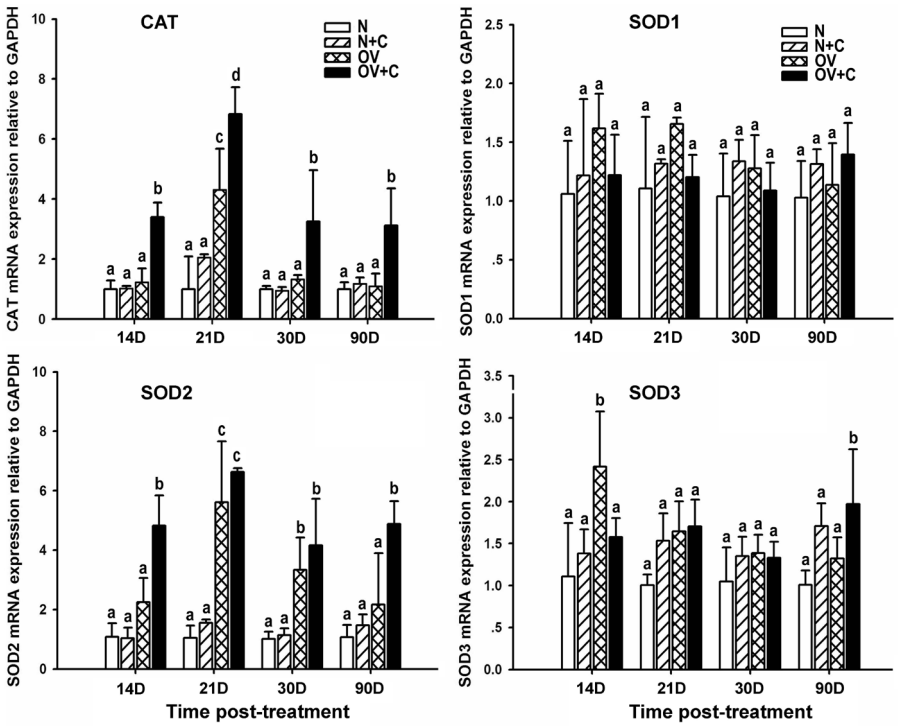

Figure 7. Effect of dietary curcumin on the expression of antioxidant genes in the liver of hamsters infected with OV. Relative mRNA expression of antioxidant genes encoding CAT, SOD1, SOD2, and SOD3 to GAPDH was assessed by quantitative RT-PCR analysis. Animals were treated with OV and/or curcumin and divided into four groups as described in Fig. 5 legend: normal hamsters fed with normal diet (N), normal hamsters and fed with 1% w/w curcumin-supplemented diet (N + Cur), OV-infected hamsters fed with normal diet (OV), and OV-infected hamsters fed with 1% curcumin-supplemented diet (OV + Cur). Data are presented as the mean ± SD of three animals and triplicate set of experiment was performed. One way ANOVA was used to compare among these four groups at each time point. a, b, c, and d are the statistical significance among all groups ($P < 0.05$). Different alphabets in the same group mean statistically significant difference in the order d > c > b > a for each time point. D = day(s).

normal hamsters with curcumin treatment compared with normal control group.

4 Discussion

We have previously reported that OV infection induces iNOS-dependent oxidative and nitrative DNA damage in experimental opisthorchiasis [9, 10], which may be involved in the initiation and promotion of CCA. Recently, we demonstrated that the level of 8-oxodG, an oxidative DNA lesion, in the urine significantly increased in the order of CCA>OV-infected patients > healthy subjects [22]. 8-oxodG level decreased after worm elimination in OV-infected patients [22] and in animal model [23]. Thus, 8-oxodG may be a useful biomarker to monitor not only infection but also carcinogenesis. In this study, we demonstrated the protective effect of curcumin, a tumeric extract, on 8-oxodG and 8-nitroguanine formation in hamsters infected with OV. Curcumin effectively suppressed oxidative and nitrative DNA damage and the expression of oxidant-generating genes such as iNOS, NF-κB, and COX-2, and enhanced the expression of antioxidant genes such as SOD2, SOD3, and CAT. Curcumin reduced OV-induced histopathological changes, including inflammatory cell infiltration and periductal fibrosis. Therefore, we hypothesize that supplement with curcumin reduces DNA damage through the suppression of inflammatory responses and balancing of oxidant–antioxidant status.

We have previously reported that OV infection strongly induces iNOS in bile duct epithelial cells and inflammatory cells [9]. Expression of iNOS is regulated by activated NF-κB translocated to the nucleus [24, 25]. Recently, we have also reported that *O. viverrini* antigen induces the expression of Toll like receptor (TLR)-2, leading to NF-κB-mediated iNOS, and COX-2 expression in macrophage cell line [26]. NF-κB is implicated in the expression of a wide variety of genes involved in inflammatory response [27]. In this study, our results showed that the expression of NF-κB, iNOS, and COX-2 decreased upon curcumin treatment especially in acute infection (days 14–21). Decreased expression of iNOS is supported by a decrease in plasma nitrate level by curcumin. Our findings are supported by a recent review on the reducing effect of curcumin on NF-κB expression [13] and related gene products including iNOS and COX-2 [24, 28]. In addition, COX-2 is a well-known

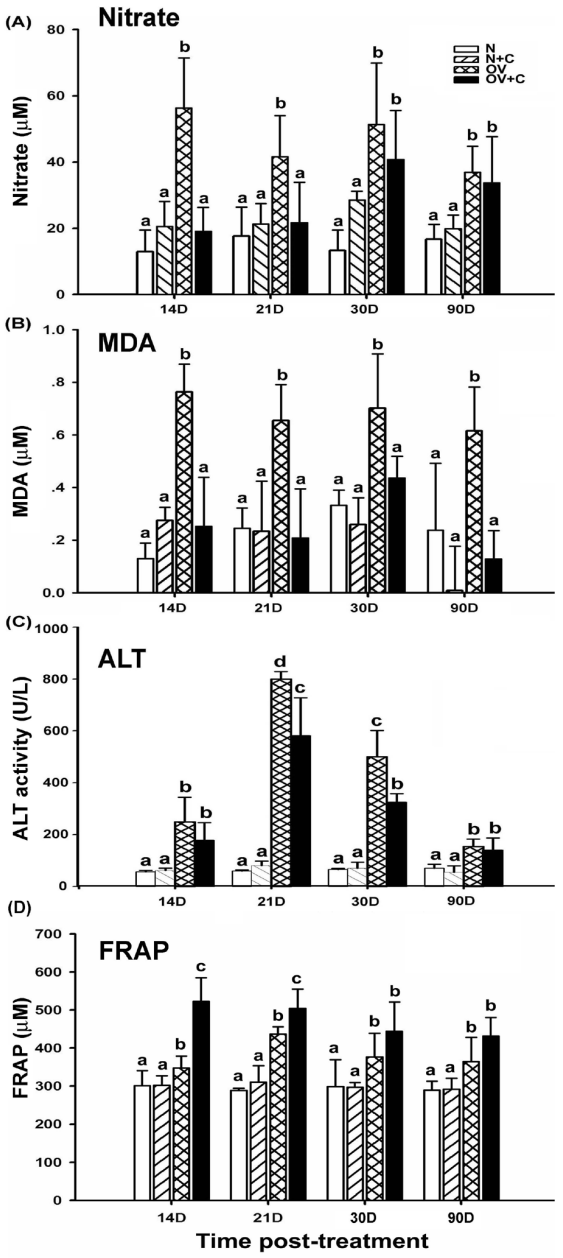

Figure 8. Effect of curcumin on the biochemical parameters in the plasma of hamsters infected with OV. Plasma levels of nitrate, MDA, ALT, and FRAP were measured as described in Materials and methods. Animals were treated with OV and/or curcumin and divided into four groups as described in Fig. 5 legend: normal hamsters fed with normal diet (N), normal hamsters and fed with 1% w/w curcumin-supplemented diet (N + Cur), OV-infected hamsters fed with normal diet (OV), and OV-infected hamsters fed with 1% curcumin-supplemented diet (OV + Cur). Data are presented as the mean ± SD of 5 animals and duplicate set of experiment was performed. One way ANOVA was used to compare among these four groups at each time point. a, b, c, and d are the statistical significance among all groups ($P < 0.05$). Different alphabets in the same group mean statistically significant difference in the order d > c > b > a for each time point. D = day(s).

mediator to inflammatory process and contributes to ROS production [29, 30], and induced by parasitic infection *via* an inflammatory reaction [26]. The inhibitory effect of curcumin on NF-κB activation might be due to suppression of IκB degradation and p65 translocation to the nucleus [31]. Our result indicates that curcumin can decrease plasma MDA, an oxidative stress marker, and plasma nitrate level in animal model of OV infection, which might explain the ability of curcumin to inhibit free radical generation and inflammation [32].

In contrast to oxidant-generating genes, we found that transcription of the antioxidant genes SOD2 and CAT increased after curcumin treatment at all time points in this study. In addition, expression of SOD3 mRNA increased only on day 90 post-treatment. Increase in the plasma level of FRAP may be supported by the transcriptional regulation of antioxidant gene expression. Curcumin enhances the expression of SOD2 and CAT for host defense against oxidative stress [13, 24, 33] by activation of the transcription factor nuclear redox factor (Nrf2) [34] leading to increase in ferric-reducing anti-oxidant power in the plasma. Increase in mitochondria SOD (SOD2) may be the first line of antioxidant defense against the overproduction of superoxide anion radical ($O_2^{\bullet -}$) during parasitic infection. It is speculated that curcumin induces over-expression of SOD2 and CAT, which partially protect the liver from injury due to increased reactive oxygen production [35]. This hypothesis is supported by the finding that curcumin supplement decreased OV-induced MDA level and ALT activity.

It has been shown that generation of $O_2^{\bullet -}$ and H_2O_2 plays an important role in host defense against parasite infection [36]. Excess NO and $O_2^{\bullet -}$ production leads to generation of highly reactive peroxynitrite ($ONOO^-$), which mediates 8-nitroguanine and 8-oxodG formation [5, 37]. Interestingly, our data showed that these DNA lesions almost completely disappeared after curcumin treatment in the liver of OV-infected hamsters. PCNA functions as a cofactor for DNA polymerase δ during DNA synthesis and repair mechanism and a marker of cell proliferation [38]. We have demonstrated that iNOS-dependent DNA damage during chronic and repeated OV infection promotes the epithelial cell proliferation, leading to cholangiocarcinogenesis [10]. This study showed that accumulation of PCNA in blie duct epithelial cells of OV-infected hamsters was dramatically reduced by curcumin, suggesting that curcumin reduces not only DNA damage but also cell proliferation to prevent cholangiocarcinogenesis.

8-Nitroguanine and 8-oxodG formation in DNA is known to cause mutation, leading to carcinogenesis. 8-oxodG formation results in G:C to T:A transversion, which is frequently found in tumor relevant genes [8, 39]. 8-Nitroguanine undergoes spontaneous depurination leading to apurinic sites in DNA [40], resulting in G:C to T:A transversion [8, 40]. These DNA lesions significantly decreased after curcumin treatment. Therefore, it can be concluded that

curcumin can inhibit nitrative and oxidative DNA damage *via* an increase in the expression of antioxidant genes and a decrease in the expression of oxidant-generating genes, reducing the risk of opisthorchiasis-associated CCA. The study therefore provides an insight into chemoprevention of OV-induced CCA.

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The authors have declared no conflict of interest.

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