CHAPTER IV
RESULTS

4.1 Effect of curcumin on inflammatory cell infiltration in *O. viverrini*-infected hamsters treated with praziquantel

To evaluate the effect of curcumin on infiltration of inflammatory cells, we performed hematoxylin and eosin staining (Figure 9). Increased eosinophil infiltration was observed after praziquantel treatment in the liver of *O. viverrini*-infected hamsters similarly to previous report (Pinlaor et al., 2008). In curcumin-treated group, at the dose of 150 mg/kg body weight, accumulation of eosinophils was significantly decreased ($P<0.05$; 8 ±3.55 *vs* 37.75 ± 7.13), while mononuclear cells, mainly lymphocytes, were significantly increased ($P<0.05$; 150.60 ± 29.38 *vs* 40.4± 5.64) compared with in *O. viverrini*-infected hamsters treated with praziquantel (OP group) as shown in Table 5.

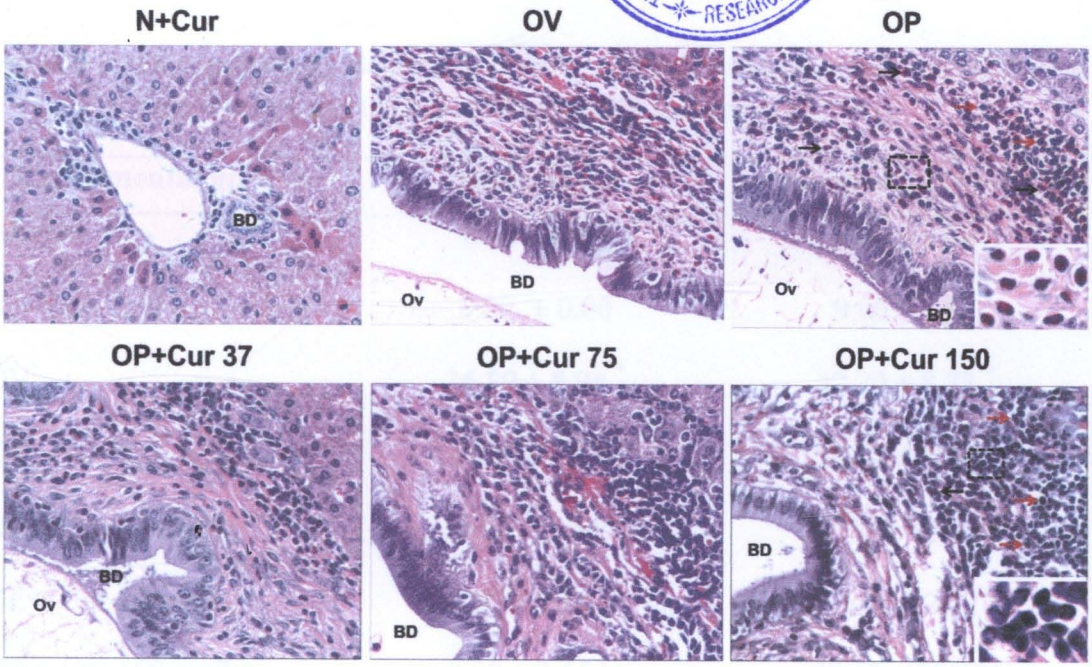

Figure 9 Histopathological changes in *Opisthorchis viverrini*-infected hamsters treated with praziquantel and the effect of a curcumin supplement. H & E staining presents the accumulation of inflammatory cells, eosinophils (black arrows), mononuclear cells (red arrows). Representative image is shown from seven animals per group. Original magnification is x400, except inset images (x1000). Ov, *O. viverrini*; BD, bile duct; N+Cur, normal hamsters supplemented with 150 mg/kg body weight of curcumin; OV, *O. viverrini* infected hamsters that received corn-oil; OP, *O. viverrini*-infected hamsters treated with praziquantel; OP+Cur 37, OP+Cur 75 and OP+Cur 150:, OP group supplemented with 37,75 and 150 mg/kg body weight of curcumin, respectively.

Table 5 Histopathology changes in *O.verrini*-infected hamsters treated with praziquentel and the effect of curcumin supplement.

Experimental groups	Eosinophils	Mononuclear cells
Normal	0.00 ± 0.00	8.00 ± 3.08
N + Cur	0.00 ± 0.00	9.00 ± 1.87
OV	36.80 ± 8.98 *	42.20 ± 8.73 *
OP	37.75 ± 7.13 *	24.88 ± 4.96 *
OP + Cur 37 mg/kg	19.00 ± 3.54 †,*	71.40 ± 10.09 †,*
OP + Cur 75 mg/kg	8.80 ± 2.86 †,*	85.80 ± 32.52 †,*
OP + Cur 150 mg/kg	8.00 ± 3.55 †,*	150.60 ± 29.38 †,*

Data are mean ± SD. $^{\dagger}P < 0.05$, compared with OP group, $^{*}P < 0.05$ compared with N group. N, Normal hamsters that received diluents; NC, Normal hamsters supplemented with curcumin 150 mg/kg; OV, *O. viverrini*-infected hamsters that received corn oil; OP, *O.viverrini*-infected hamsters treated with praziquentel; OP + Cur, *O. viverrini*-infected hamsters treated with praziquantel and supplemented with curcumin. The number of eosinophils and mononuclear cells was assessed in 10 randomly selected fields (400x) per section by three blinded observers from the author.

4.2 Effect of curcumin on Nrf2 expression in *O. viverrini*-infected hamsters treated with praziquantel

To assess the role of Nrf2-mediated process in stress response, we examined its localization in the liver of *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement (Figure 10). In curcumin-treated group, an immunoreactivity of Nrf2 was observed at the nucleus of inflammatory cells and bile duct epithelial cells. At a dose of 150 mg/kg body weight, expression of Nrf2 was significantly increased compared with OP group ($P<0.05$; 167.4±39.85 *vs* 103.4 ± 20.99, Figure 10A). Its localization was associated with mononuclear cell infiltration ($R = 0.73$, $P = 0.001$). To determine protein expression level of Nrf2, we performed Western blot analysis. Curcumin treatment dose-dependently increased the expression level of Nrf2 in the cytoplasm and in the nuclear extract. A significant increase in cytoplasm and nuclear Nrf2 expression was observed at a dose of 150 mg/kg body weight compared with OP group ($P<0.05$, Figure 10B and C). This was correlated with mRNA expression level evaluated by real-time RT-PCR (Figure 10D). In addition, expression level of Nrf2 in normal hamsters was similar to those receiving diluent.

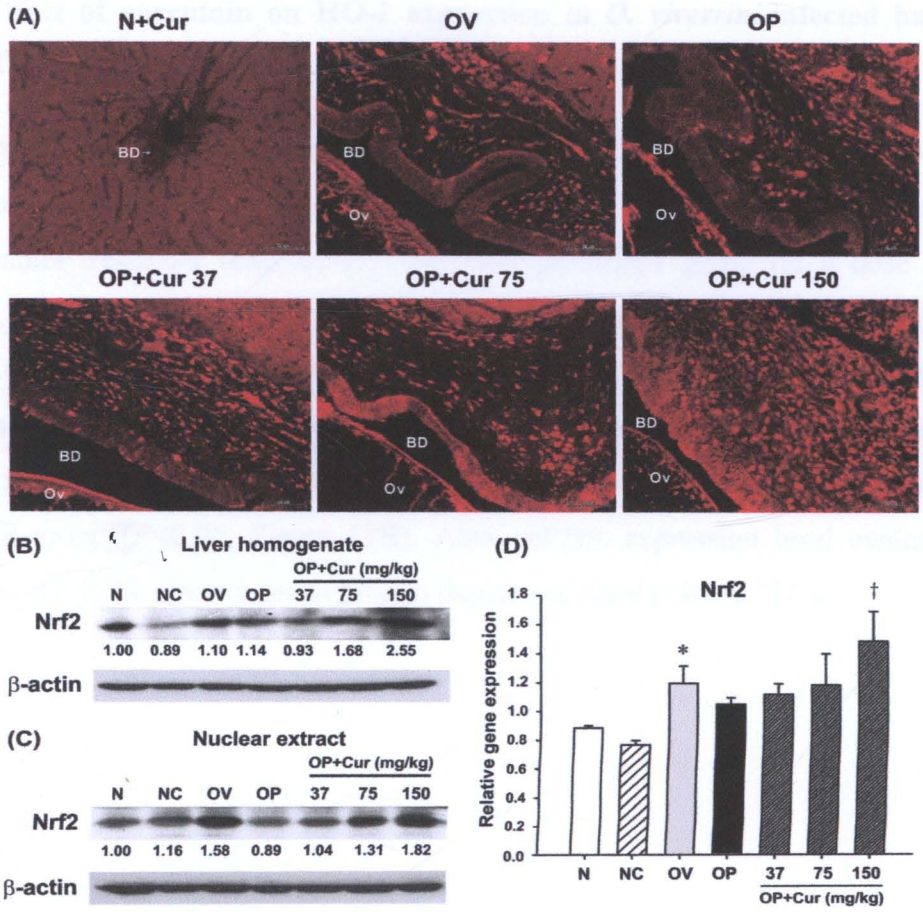

Figure 10 Expression of Nrf2 in *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement. (A) Fluorescent immunohistochemical staining of Nrf2 after curcumin supplement in *O. viverrini*-infected hamsters with praziquantel treatment. Original magnification is x400. (B) Liver homogenate and (C) nuclear protein expression of Nrf2 was analyzed by Western blot. The number below each photograph indicates the fold increase in protein expression relative to normal control (N). (D) mRNA level of Nrf2 relative to GAPDH was measured by real-time RT-PCR. The statistical significance was analyzed using One-Way ANOVA of 3 hamsters in each group. Data are expressed as fold change over normal hamsters that received diluent (mean ± SD of duplicate independent experiments). *$P<0.05$, compared to N group, † $P<0.05$, compared to OP group. Abbreviations are the same as in Figure 9 legend.

4.3 Effect of curcumin on HO-1 expression in *O. viverrini*-infected hamsters treated with praziquantel

Immunohistochemical analysis showed intense immunoreactivity of HO-1 as an indicator of antioxidant defense and the immunoreactivity was decreased by praziquantel treatment (Figure11A). In curcumin-treated group (at a dose of 150 mg/kg), expression of HO-1 was significantly higher than in OP group ($P<0.05$; 1.80±0.45 *vs* 1.20±0.45, Figure 11A). This result was confirmed by Western blot analysis (Figure 11B). Expression of HO-1 in curcumin-treated group at a dose of 150 mg/kg of curcumin, a significant increase in HO-1 expression was observed compared with OP group ($P<0.05$, Figure 11B). Also, mRNA expression level evaluated by real-time RT-PCR was similar to protein expression level (Figure 11C).

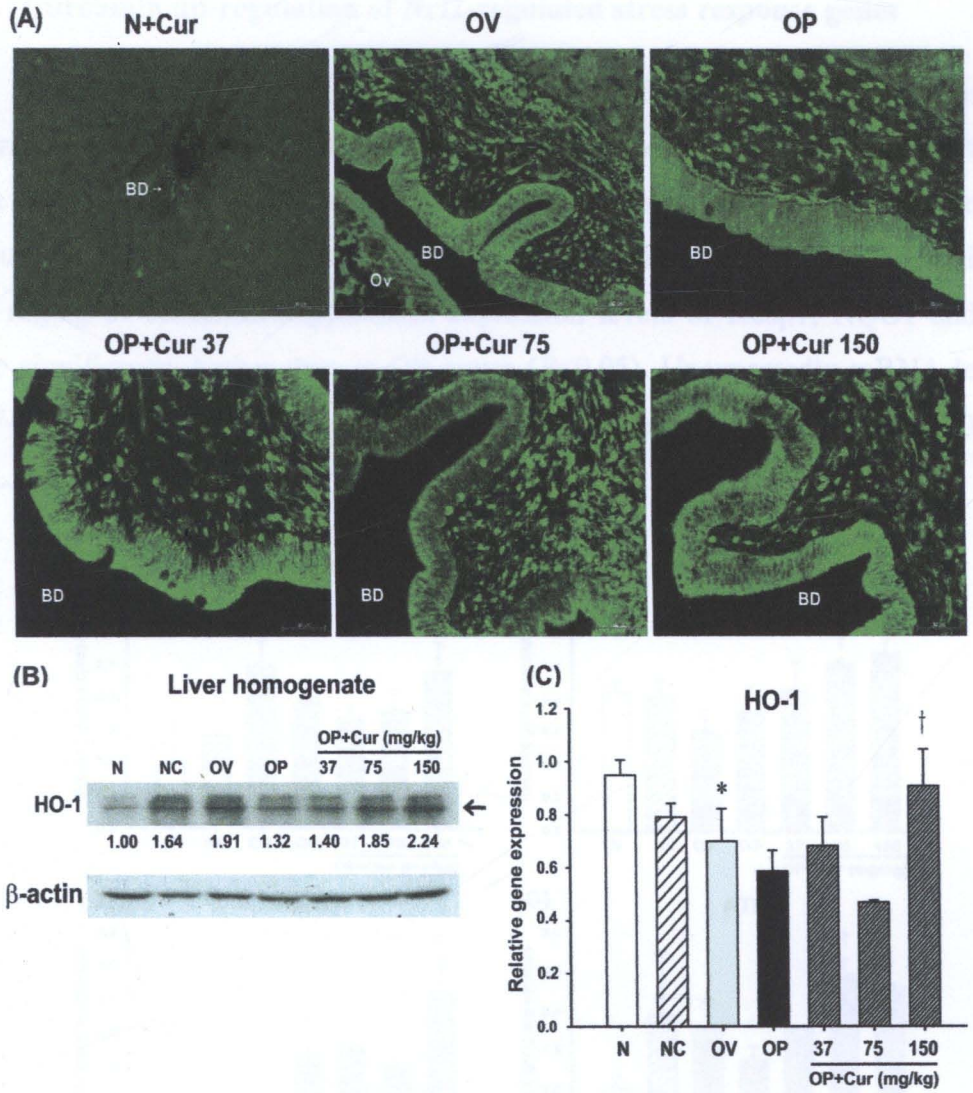

Figure 11 Expression of HO-1 in *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement. (A) Fluorescent immunohistochemical staining of HO-1 after curcumin supplement in *O. viverrini*-infected hamsters with praziquantel treatment. Original magnification is x400. (B) Protein expression level of HO-1 was measured by Western blot. (C) mRNA level of HO-1 relative to GAPDH was measured by real-time RT-PCR. Data are expressed as fold change over normal hamsters that received diluent (mean ± SD of duplicate independent experiments, n=3). *$P<0.05$, compared to N group, † $P<0.05$, compared to OP group. The statistic and abbreviations are the same as in Figure 9 legend.

4.4 Curcumin up-regulation of Nrf2-regulated stress response genes

To investigate whether curcumin induces Nrf2-mediated stress response, we determined Keap1, NQO1, and GCL genes in *O. viverrini*-infected hamsters treated with praziquantel by real-time RT-PCR (Figure 12). mRNA levels of these genes in curcumin-treated group tended to increase in a dose-dependent manner. At a dose of 150 mg/kg of curcumin supplement, expression levels of Keap1, NQO1 and GCL were significantly higher than in OP group ($P<0.05$). Unexpectedly mRNA level of ATF-3 in curcumin-treated group also increased similarly to Nrf2, Keap1, NQO1, and GCL.

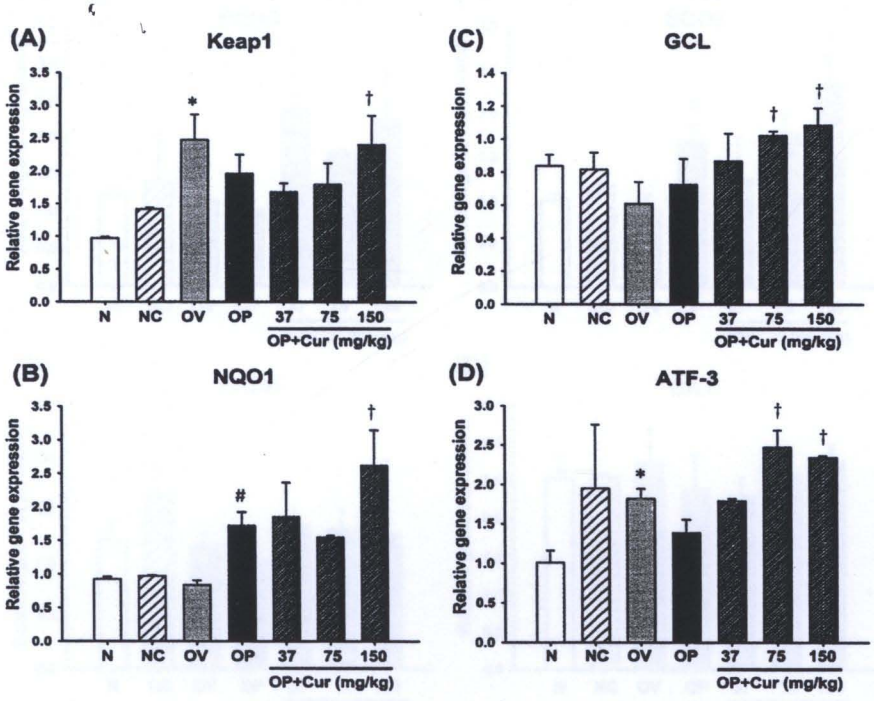

Figure 12 mRNA levels of Nrf2-regulated stress response genes in *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement. mRNA levels of Keap1, NQO1, GCL and ATF-3 relative to GAPDH were evaluated by real-time RT-PCR. Data are expressed as fold change over normal hamsters that received diluent (mean ± SD of duplicate independent experiments, n=3). *$P<0.05$, compared to N group, #$P<0.05$, compared to OV group, † $P<0.05$, compared to OP group. The statistic and abbreviations are the same as in Figure 9 legend.

4.5 Effect of curcumin on the expression of antioxidant genes

The effect of curcumin treatment on the expression of antioxidant genes in *O. viverrini*-infected hamsters treated with praziquantel. As shown in figure 13, the mRNA levels of Prdx3, Prdx6, SOD2, and CAT genes in curcumin-treated group tended to increase compared with OP group. A dose-dependent increase was observed with SOD2 and CAT expression. At a dose of 150 mg/kg of curcumin supplement, mRNA levels of Prdx3 and SOD2 were significantly higher than in OP group ($P<0.05$).

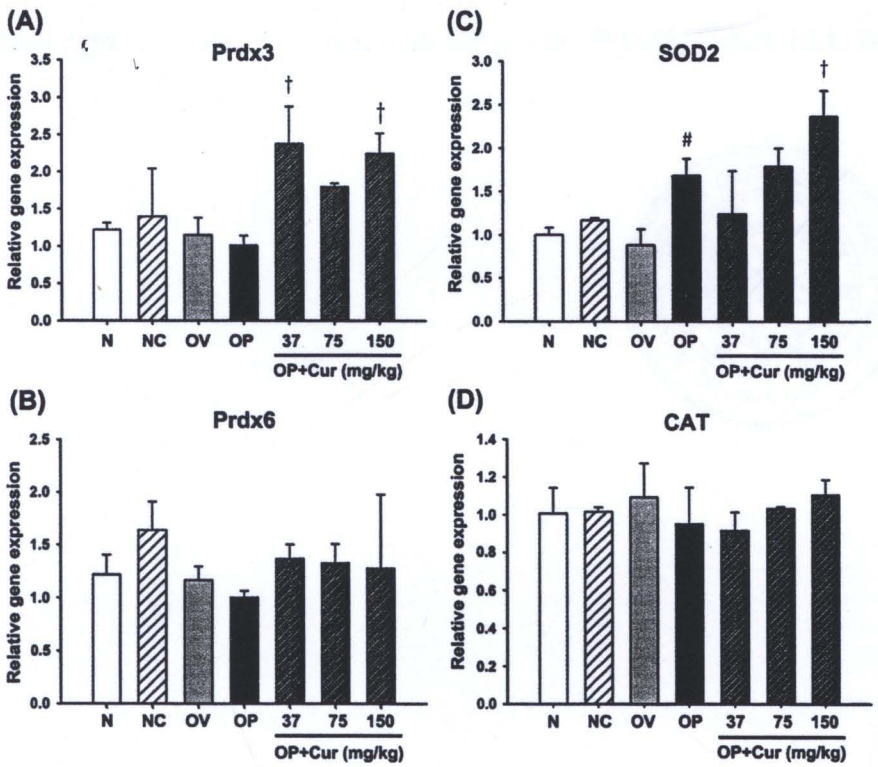

Figure 13 mRNA levels of antioxidant genes in *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement. mRNA levels of Prdx3, Prdx6, SOD2 and CAT relative to GAPDH were evaluated by real-time RT-PCR. Data are expressed as fold change over normal hamsters received diluent (mean ± SD of duplicate independent experiments, n=3). # $P<0.05$, compared to OV group. † $P<0.05$, compared to OP group. The statistic and abbreviations are the same as in Figure 9 legend.

4.6 Effect of curcumin on the expression of oxidant genes

Figure 14 showed the effect of curcumin treatment on oxidant genes in *O. viverrini*-infected hamsters treated with praziquantel. Real-time RT-PCR analysis revealed that mRNA expression levels of NF-κB (Figure 14C), iNOS (Figure 14E), and COX-2 (Figure 14F) in curcumin-treated group tended to decrease in a dose-dependent manner. To evaluate the protein expression level, we performed by Western blot analysis. NF-κB levels in cytoplasm and nucleus were similar to mRNA expression, and iNOS expression was also in agreement with its transcriptional level. Notably, a dose of 150 mg/kg body weight of curcumin significantly decreased of NF-κB and iNOS protein levels compared with OP group ($P<0.05$, Figure 14A, B and D).

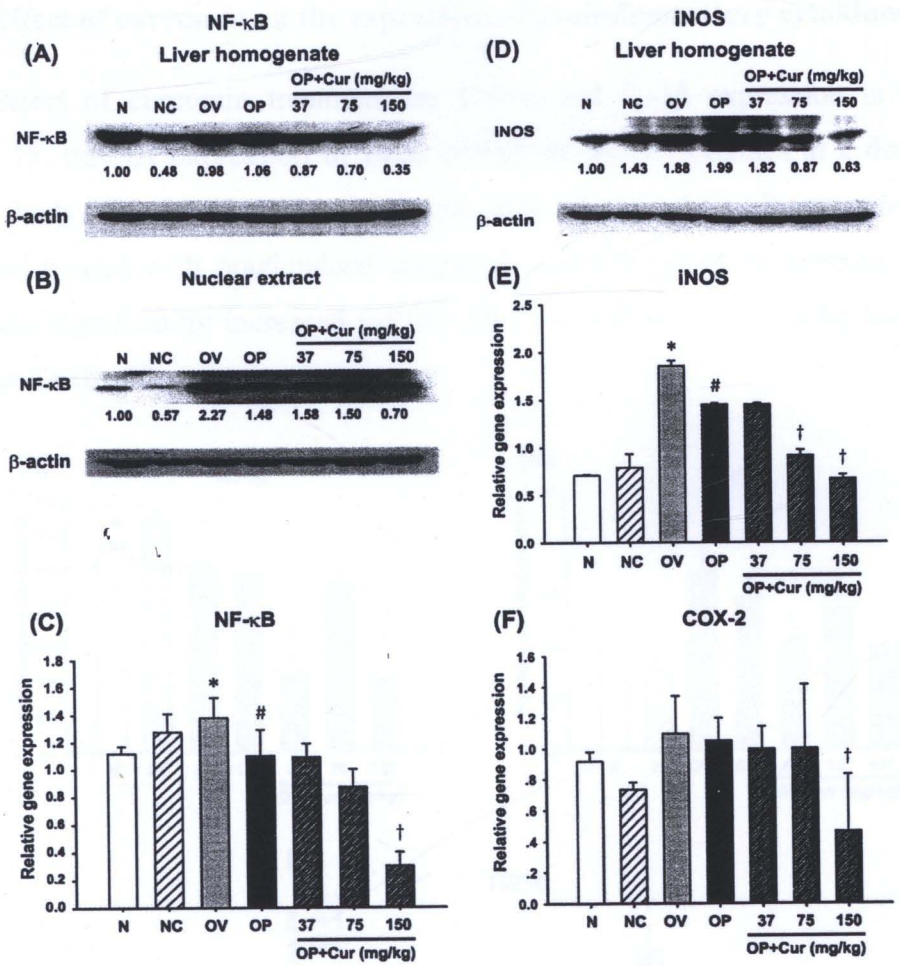

Figure 14 Expression levels of oxidant genes in *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement. Western blot was used to evaluate the expression of NF-κB in (A) liver homogenate and (B) nuclear extract, and (D) expression level of iNOS in liver homogenate. The number below each photograph indicates the fold increase in the levels of protein expression relative to normal control. mRNA levels of (C) NF-κB, (E) iNOS, and (F) COX-2 relative to GAPDH were evaluated by real-time RT-PCR. Data are expressed as fold change over normal hamsters that received diluent (mean ± SD of duplicate independent experiments, n=3). **P*<0.05, compared to N group, # *P*<0.05, compared to OV group, † *P*<0.05, compared to OP group. The statistic and abbreviations are the same as in Figure 9 legend.

4.7 Effect of curcumin on the expression of proinflammatory cytokines

Effect of curcumin treatment on TNF-α and IL-1β expression is shown in Figure 15. mRNA expression of these proinflammatory cytokines at a dose of 150 mg/kg body weight of curcumin significantly decreased in *O. viverrini*-infected hamsters treated with praziquantel compared with OP group. In contrast, curcumin treatment significantly increased mRNA level of TGF-β at 150 mg/kg body weight compared with OP group.

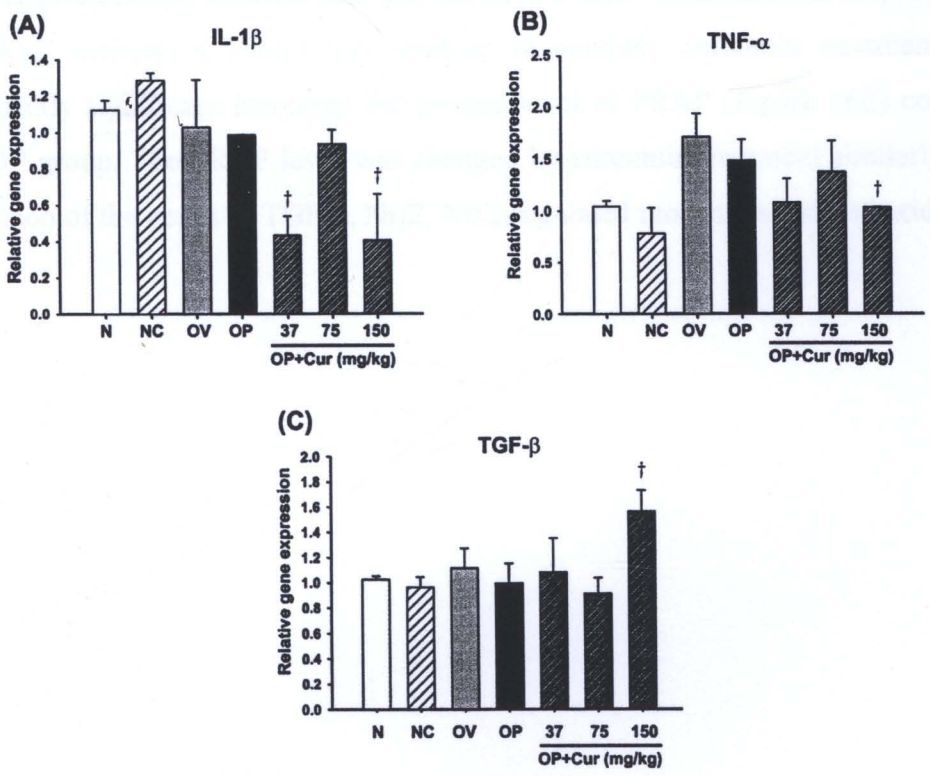

Figure 15 mRNA levels of proinflammatory cytokines in *O. viverrini*-infected hamsters treated with praziquantel and the effect of curcumin supplement. mRNA expression of (A) IL-1β, (B) TNF-α , and (C) TGF-β relative to GAPDH were evaluated by real-time RT-PCR. Data are expressed as fold change over normal hamsters that received diluent (mean ± SD of duplicate independent experiments, n=3). *$P<0.05$, compared to N group, † $P<0.05$, compared to OP group. The statistic and abbreviations are the same as in Figure 9 legend.

4.8 Effect of curcumin on biochemical parameters in *O. viverrini*-infected hamsters treated with praziquantel

Figure 16 shows the effect of praziquantel treatment on urinary 8-oxodG level (Figure 16A), plasma levels of nitrate/nitrite (Figure 16B), MDA (Figure 16C), ALT (Figure 16D) and FRAP (Figure 16E) in *O. viverrini*-infected hamsters treated with praziquantel. After praziquantel treatment, the levels of 8-oxodG, nitrate/nitrite, MDA and ALT tended to increase compared with OV group. Curcumin treatment decreased the level of 8-oxodG, nitrate/nitrite and MDA in a dose-dependent manner, in parallel with ALT activity, a liver injury marker. In contrast, curcumin treatment dose-dependently significant increased the plasma level of FRAP (Figure 16E) compared with OP group. The FRAP level was changed by curcumin treatment similarly to the expression of the genes of TGF-β, Nrf2, Nrf2-regulated molecules and antioxidants.

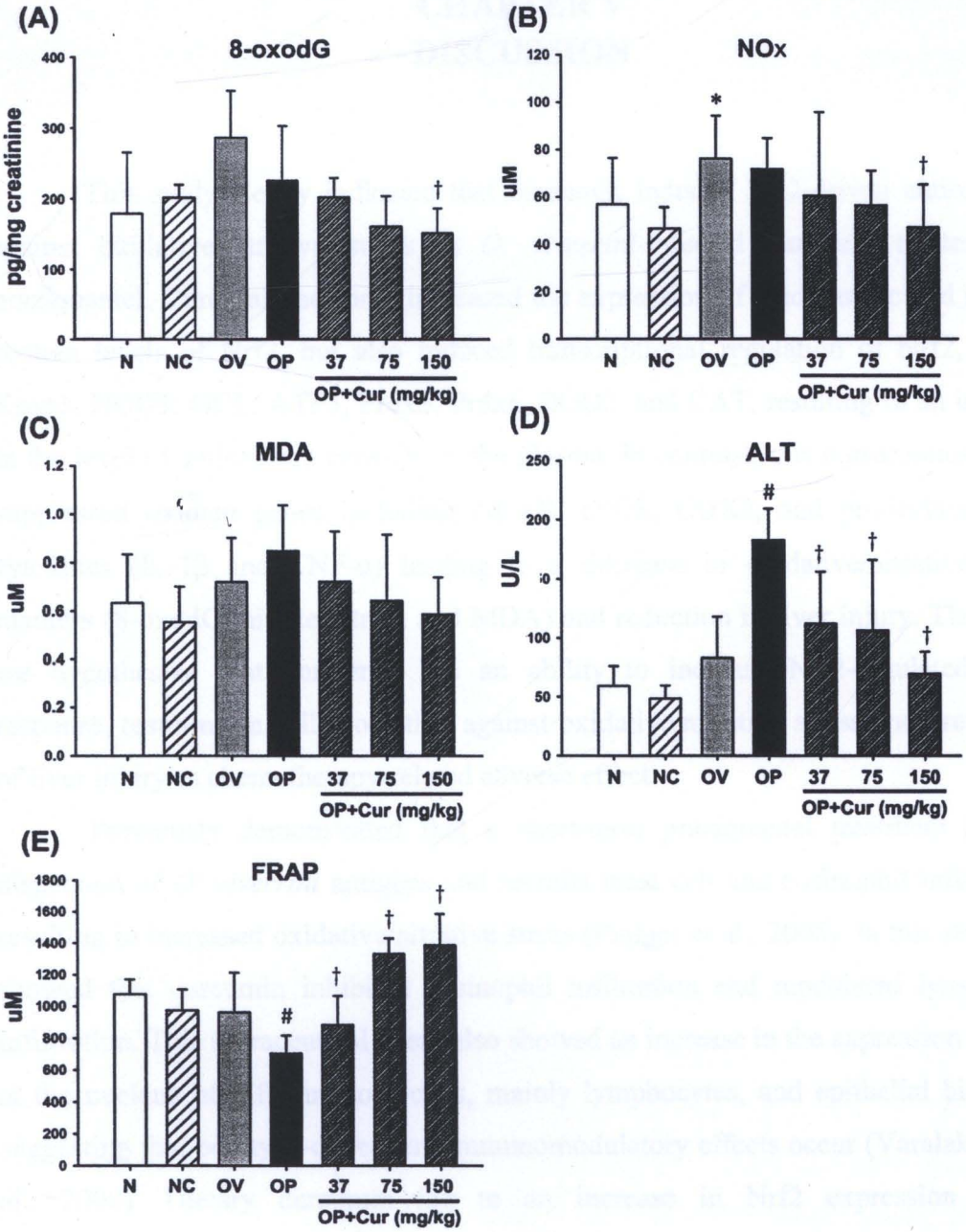

Figure 16 Effect of curcumin on biochemical parameters in *O. viverrini*-infected hamsters treated with praziquantel. (A) Urinary levels of 8-oxodG, and plasma levels of (B) nitrate/nitrite, (C) MDA, (D) ALT activity, and (E) FRAP. Each value represents as mean ± SD of duplicate independent experiments (n=7). *P<0.05, compared to N group, # P<0.05, compared to OV group, † P<0.05, compared to OP group. The abbreviations are the same as in Figure 9 legend.