CHAPTER III
RESEARCH METHODOLOGY

3.1 Parasites

O. viverrini metacercariae were obtained from naturally infected cyprinoid fishes. The cyprinoid fishes were collected from endemic areas in Khon Kaen province. The fishes were digested by artificial 0.25% pepsin-HCl by using electric blender. The ratio of fishes: pepsin A solution was 1: 3 by volume. The mixture was incubated at 37°C in the shaking water bath for 1 hour. The digested content was filtered, washed and resedimented several times, then the sample was collected in sediment jar. The metacercariae of *O. viverrini* were isolated under a dissecting microscope. Only the viable active cysts were used to infect hamsters.

3.2 Hamster infection

Fifty metacercariae of *O. viverrini* were given per hamster via gastric or stomach intubation. A gastric tube was gently inserted through the oral cavity into the esophagus until reaching a stomach. A syringe contain 50 metacercariae in approximately 1 ml of NSS connecting to a blunted end needle were then carefully deliver.

3.3 Animal and experimental design

The study protocol has been approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 46/2552). Four to six-week-old male golden hamsters were housed under conventional conditions and fed with a stock murine diets (CP-SWT, Thailand) were given water *ad libitum*. Seven animals were included in each of the following groups:

Group 1 normal hamsters

Group 2 normal hamsters and supplemented with curcumin

Group 3 *O. viverrini*-infected hamsters and supplemented with diluents

Group 4 *O. viverrini*-infected hamsters treated with praziquantel

Group 5 *O. viverrini*-infected hamsters treated with praziquantel and supplemented with curcumin at the concentration 37 mg/kg body weight

Group 6 *O. viverrini*-infected hamsters treated with praziquantel and supplemented with curcumin at the concentration 75 mg/kg body weight

Group 7 *O. viverrini*-infected hamsters treated with praziquantel and supplemented with curcumin at the concentration 150 mg/kg body weight

In *O. viverrini* infected hamsters, each animal was infected with 50 metacercaria of *O. viverrini.* After 3 months of post-infection period, hamsters were treated by oral curcumin daily for 2 weeks. After that infected hamsters were treated with a single dose of praziquentel (400 mg/kg body weight) and were sacrificed at 12 hours after treatment, the period which have showed the maximal oxidative and nitrative stress as reported previously (Pinlaor et al., 2008).

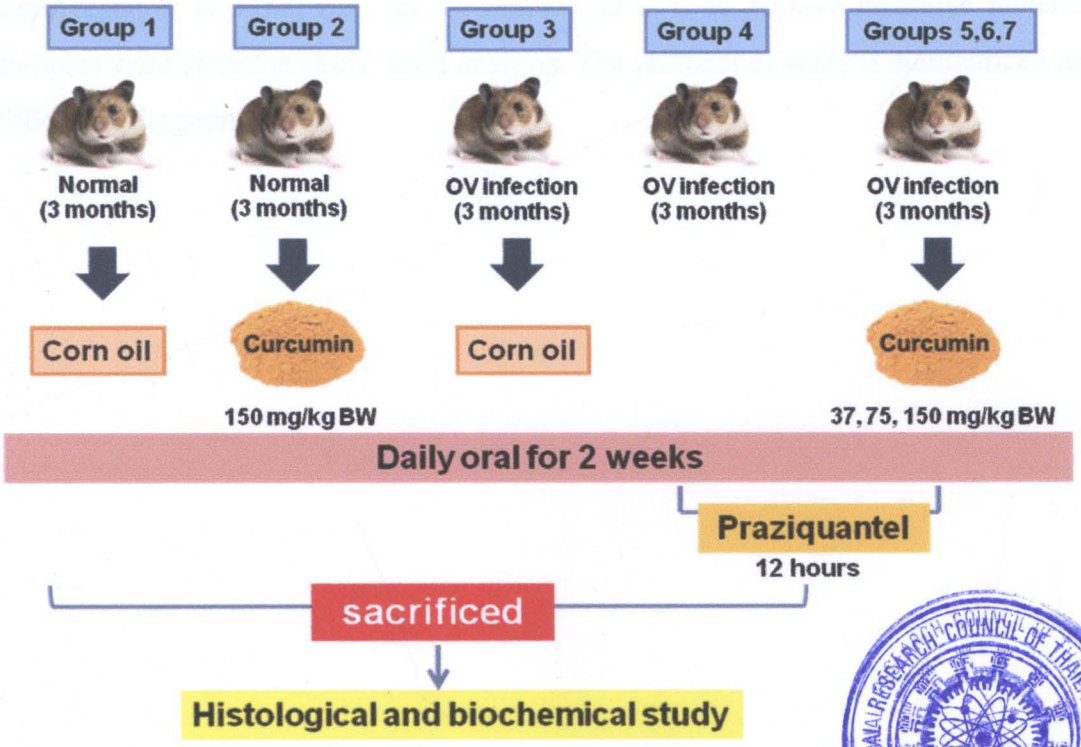

Figure 6 The experimental design of animal model

3.4 Specimen collection and protocol of the study

After the period of treatment, hamsters were anesthetized with ether and then liver tissues were collected. The liver sections of 0.5 cm in diameter (approximately 150 mg) were dissected from the hilar region and adjacent areas including the secondary order bile duct lumens, where the worms were usually found, and use for either total RNA isolation or histopathological study. For total RNA isolation, the livers were immediately treated with TRIZOL reagent and then store 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 3,000 rpm for 10 minutes, plasma was collected and store at -80°C until analysis. Approximately, 100-200 µl of urine from urinary bladder was also collected on the day after of scarification. After centrifugation at 3,000 rpm for 10 minutes at 4 °C to remove insoluble meterials samples were stored at -80°C until analysis. The protocol of study is summarized as a following diagram.

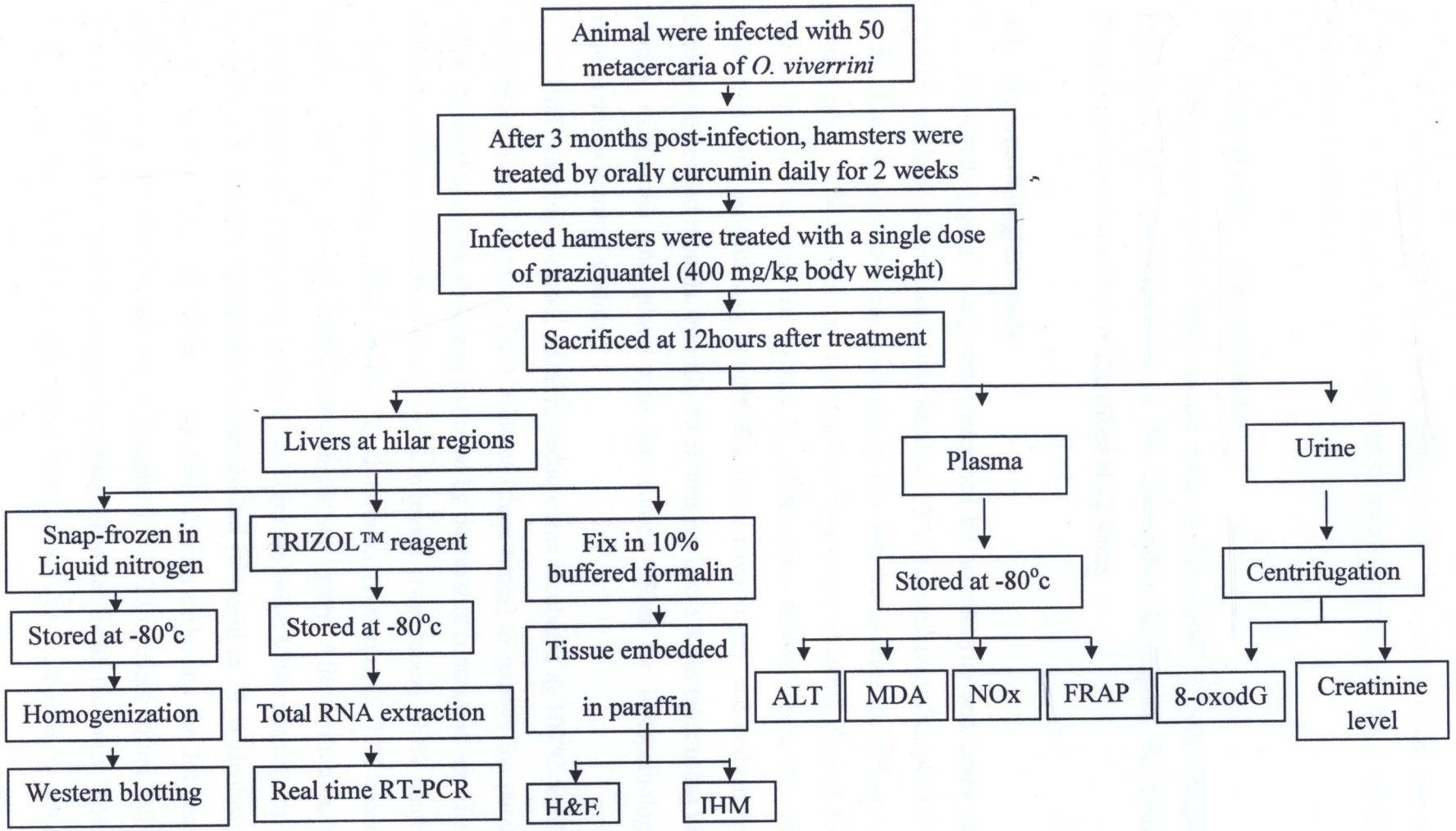

Figure 7 Protocol of specimen collection and analysis

3.5 Administration of curcumin

Curcumin was dissolved in a small amount of corn oil (final concentration: <1%). Curcumin (or corn oil) was orally administered daily for 2 weeks before praziquantel treatment.

3.6 Administration of praziquantel

Praziquantel (Biltricide®, single dose of 400 mg/kg of body weight; Bayer, Pittsburgh, PA) was suspended in 2% Cremophor EI (Sigma, St. Louis, MO). Praziquantel was treated before sacrificed at 12 hours.

3.7 Histopathological study

Histopathological study was performed by hematoxylin and eosin staining in paraffin sections. Liver tissue was fixed in 10% buffered formalin, pH 7.0 for 6-12 hours. After fixation process, liver tissue was dehydrated through 70% alcohol for 1 (two times), 95% alcohol for 1hour (three times) and absolute alcohol for 1 hour (three times), and clear in xylene for 1hour (two times). Then, the tissue was infiltrated with paraffin wax at 60°C for 1hour (two times), and embeds by placing them in embedded moulds. Paraffin block was cut into a 5 μm thick using Lietz 1512 Rotary microtome and place onto the coated slides for histopathological and immunohistochemical studies.

H&E staining method, paraffin section was incubated at 100°C for 3 minutes and deparaffinize in xylene for 3 minutes (three times) to remove the paraffin wax. After deparaffinization, slide was hydrated by decreasing concentrations of ethanol as following steps; absolute alcohol for 2 minutes (two times), 90% alcohol for 2 minutes (two times), 80% alcohol for 2 minutes (two times), 70% alcohol for 2 minutes (two times) and finally rinse in the tap water. After hydration, slide was stained with Harr's hematoxylin for 10 minutes and wash in the running tap water for 2 minutes. Following by destain in acid alcohol (1% acid in 70 % alcohol), wash in the running tap water and blue in saturated lithium carbonate for 3-4 seconds was performed. After that, it was washed again for 10-20 minutes before staining with eosin solution for 15-20 seconds and shaking the slides for all the times. The slide was then being dehydrated through repeat three times for 3 minutes in each step as

following: 70%, 80%, 95% and absolute alcohol, xylene solution, and mount. The appropriate result for nuclei was stained blue and cytoplasm was stained red color.

3.8 Immunohistochemical study

To investigate Nrf2 and HO-1 formation, hamster liver were assessed by immunofluorescence labeling. 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 autoclave at 110°C for 10 minutes for antigen unmasking. Next, the sections were incubated with 1% skim milk for 30 minutes. Then, the sections were incubated with rabbit polyclonal anti-Nrf2 antibody and goat polyclonal anti-HO-1 antibody overnight at room temperature (each 1:100, Santa Cruz Biotechnology). Finally, they were incubated with Alexa 594-labeled goat antibody against rabbit IgG (1:400, Invitrogen) and Alexa 488-labeled donkey antibody against goat IgG (1:400, Invitrogen) for 2 hours. The stained sections were examined using a fluorescence microscope.

3.9 Determination of biochemical parameters

3.9.1 8-OH-dG levels in urine determination

Urinary 8-hydroxy-2-deoxy guanosine (8-OH-dG) levels were measured by using an ELISA kit (Japan Institute for the Control of Aging, Japan). Sample preparation and the procedure of assay were performed according to the manufacturer's instructions and the data were expressed as pg/mg creatinine. This assay was base on the competition between 8-OH-dG and 8-OH-dG-acetylcholinesterase (AChE) conjugate (8-OH-dG tracer) for a limited amount of 8-OH-dG monoclonal antibody. Because the concentration of the 8-OH-dG Tracer is held constant while the concentration of 8-OH-dG varies, the amount of 8-OH-dG Tracer that able to bind to the 8-OH-dG monoclonal antibody was inversely proportional to the concentration of 8-OH-dG in the well. This antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate was washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) was added to the well. The product

of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The product of this enzymatic color, determined spectrophotometrically, was proportional to the amount of 8-OH-dG Tracer bound to the well, which was inversely proportional to the amount of free 8-OH-dG present in the well during the incubation; or Absorbance α [Bound 8-OH-dG Tracer] α 1/ [8-OH-dG]. A schematic of this process was shown in Figure 8.

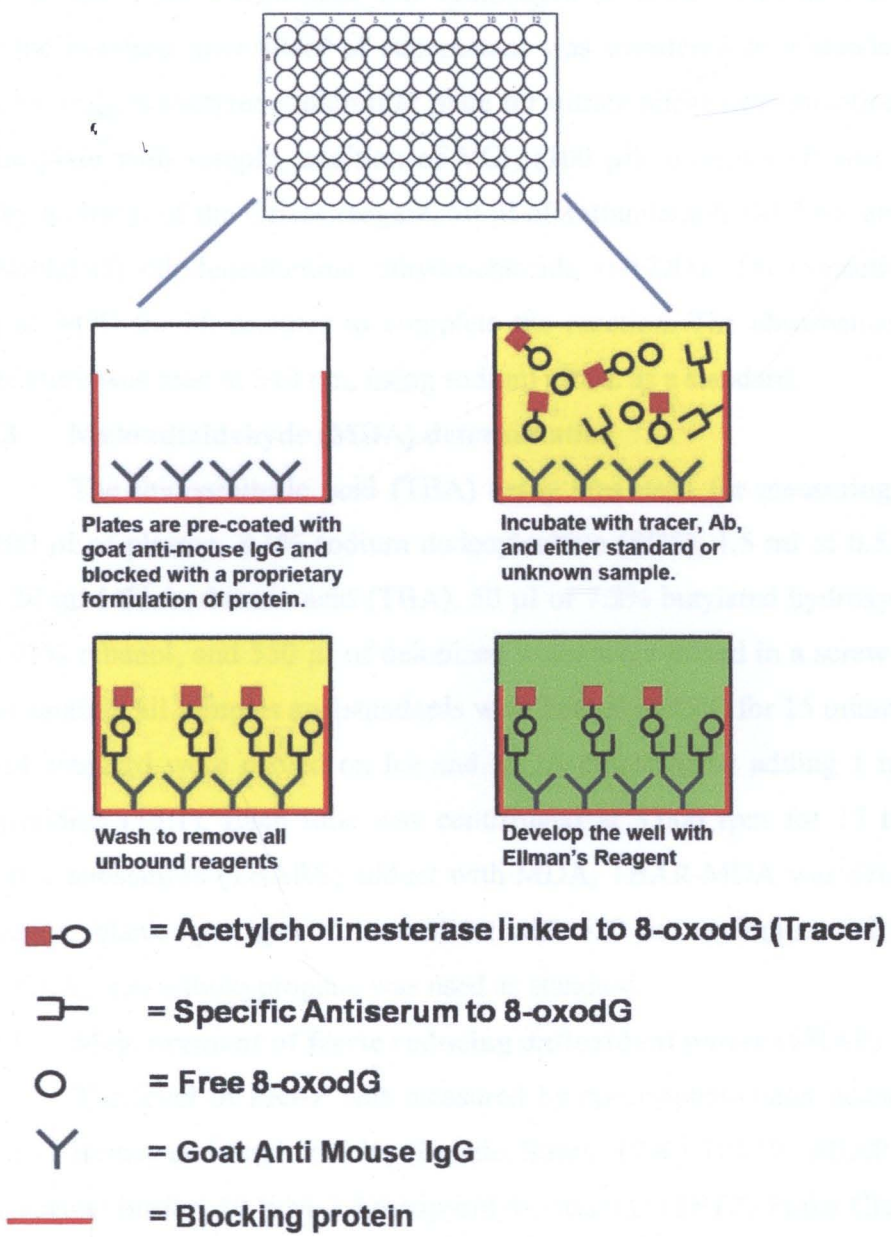

Figure 8 Schematic of 8-OH-dG determination by ACE™ EIA

3.9.2 Nitrate/nitrite (NOx) determination

The amount of NO production was determined as total nitrate/nitrite concentration in the plasma by the simple Griess reaction (Miranda et al., 2001). The nitrate concentration in the biological samples was measured after catalyzed reduction to nitrite using vanadium (III) chloride (VCl_3) and reacted with the Griess reagent. Briefly, one hundred microliters of liver homogenate or plasma was deproteinized by adding 200 microliters of methanol: diethyl ether (3: 1 mixture v/v), followed by incubation at -80°C for 30 minutes and centrifuged at 12,000 rpm at 4°C for 10 minutes. One hundred microliters of supernatant was transfered to a standard flat-bottomed, 96-well, polystyrene microtiter plate for nitrate/nitrite determination. After loading the plate with sample, addition of VCl_3 (100 µl) to each well was rapidly followed by addition of the Griess reagent, 50 µl of sulfanilamide (SULF) and 50 µl of *N*-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD). Then reaction was incubated at 34°C for 20 minutes to complete the reaction. The absorbance of the reaction mixture was read at 540 nm, using sodium nitrite as a standard.

3.9.3 Malondialdehyde (MDA) determination

The thiobarbituric acid (TBA) assay was used for measuring MDA. Briefly, 200 µl of plasma, 8.1% sodium dodecylsulfate (SDS), 1.5 ml of 0.5M HCl, 1.5 ml of 20 mM thiobarbituric acid (TBA), 50 µl of 7.2% butylated hydroxytoluene (BHT) in 95% ethanol, and 550 µl of deionized water were mixed in a screw-capped tube. After mixing, all samples and standards were heated at 95°C for 15 minutes. The sample and standard were cooled on ice and MDA extracted by adding 1 ml of n-butanol: pyridine (15:1). Each tube was centrifuged at 3,000 rpm for 15 minutes. TBA-reactive substances (TBARS) adduct with MDA, TBAR-MDA was determined against reagent blank by a spectrofluorometer with 520 nm excitation and 550 nm emission. 1,1,3,3-tetraethoxypropane was used as standard.

3.9.4 Measurement of ferric reducing antioxidant power (FRAP)

The level of FRAP was measured by spectrophotometer according to the method of Benzie and Strain (1996) (Benzie, Strain, 1996). Briefly, FRAP reagent [300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka Chemicals, Switzerland), and 20 mM ferric chloride ($FeCl_3.6H_2O$), ratio 10:1:1] were incubated

with 20 µl of plasma or standard calibrator ($FeSO_4.7H_2O$) at room temperature for 10 minutes. The absorbance was measured at 593 nm.

3.9.5 Liver function enzyme activity assay

Plasma alanine aminotransferase (ALT) activity was analyzed by a spectrophotometer (automate RA100) using a commercial kit (Thermo Trace Ltd., Australia). Conformance biochemistry control was used as the standard of this enzyme.

3.10 Gene expression study

3.10.1 Total RNA extraction

Total RNA was isolated from liver tissue (approximately 150 mg) following the manufacturer's protocol of Trizol Reagent (Invitrogen, USA). In brief, 1.5 ml of Trizol Reagent was added into tissue and homogenized using glass homogenizer. The lysate was then incubated at room temperature for 10 minutes to permit the complete dissociation of nucleoprotein complex and centrifuged at 12,000 rpm at 4°C for 10 minutes. 1,000 µl of the supernatant was then transferred to new 1.5 ml microtube. 0.3 ml of chloroform was added and shaked tube vigorously by hand for 15 seconds, followed by incubated at room temperature for 3 minutes. The mixture was centrifuged at 12,000 rpm at 4°C for 15 minutes. After centrifugation, aqueous phase was transferred to new 1.5 ml microtube. The RNA was precipitated from the aqueous phase by mixing with 0.75 ml of isopropanol. The sample was incubated at room temperature for 10 minutes and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant was removed. The RNA pellet was washed once with 75% ethanol and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant was discarded and air-dried the pellet for 5-10 minutes. The pellet was then re-dissolved with 210 µl of RNase-free water by vortex briefly.

3.10.2 Reverse Transcription

Reverse transcription reaction consisted of 3 µg of total RNA and 0.5 µg of random hexamer in total volume of 10 µl in RNase-free water, which was mixed together and then incubated at 70°C for 10 minutes. The tube was cooled immediately on ice to prevent secondary structure from reforming. The following components were added to the annealed primer/template in the order shown.

M-MLV 5x Reaction buffer	5 µl
10 mM dNTP mix	3 µl
Recombinant RNasin® Ribonuclease Inhibitor	20 units
M-MLV RT	200 units
Nuclease-Free water to final volume	25 µl

The reaction was mixed gently by flicking the tube and incubated at 25°C for 10 minutes, at 42°C for 60 minutes and 70°C for 10 minutes respectively and stored at -20°C for further study.

3.10.3 Primers for real time RT-PCR analysis

The Oligonucleotide primer pair for relative gene expression was designed base on RNA sequences of rat, mouse and hamster from Genbank by using Primer 3 Software (Table 3). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous controls. All primer sets have a calculated annealing temperature of 55°C.

3.10.4 Real-time RT-PCR analysis

Relative mRNA expression was performed by ABI7500 thermal cycler (Applied Biosystems) using a SYBR Green assay. The PCR cycling condition was 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. After PCR, a dissociation curve was constructed in the range of 60°C to 99°C according to the dissociation protocol of the instrument. All data were analyzed using Rotor Gene 5 software (Corbett Research, Australia) with a cycle threshold (Ct) in the linear range of amplification and then processed by the $2^{-\Delta\Delta Ct}$ method. Relative mRNA expression was calibrated using expression of GAPDH gene and reported as fold change over background levels in the normal control sample.

3.10.5 Cloning of PCR product

To confirm the identity of the PCR fragment, RT-PCR was performed using a Thermocycler (GeneAmp®, PCR system 9700). Fragment identity was confirmed after cloning into a home-made T-vector, followed by sequencing using the respective gene-specific primers with the Cy5-labeled primer (Applied Biosystems, USA) on MegaBACE™ 1000 DNA analysis System (Pharmacia, USA). The DNA sequence was analyzed using BioEdit software (http://www.mbio.ncsu.edu/BioEdit). The nucleotide and protein database at the National Center for Biotechnology

Information was searched using the BLAST algorithm (http://www.ncbi.nlm. nih.gov./BLASTn or BLASTX). Sequences of amplified fragment identities to rat, mouse and human nucleotides and amino acids are shown in Table 4.

3.11 Western blot analysis

3.11.1 Preparation of nuclear extract and homogenate livers

To prepare the whole tissue lysis proteins, liver tissues (75–100 mg) were homogenized in ice-cold extraction buffer (50 mM Tris-HCl pH 7.4, 0.1% SDS, 1% Triton 125 X-100, 150 mM NaCl2). The suspension was centrifuged, and the supernatant was collected and stored at -80°C until use. Nuclear extracts was prepared as described previously (Singh, Aggarwal, 1995). Briefly, liver tissues (75–100 mg) was minced using a glass homogenizer in ice-cold lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 0.5% NP-40, 0.5 mM PMSF]. After centrifugation, the resulting nuclear pellet was suspended in nuclear extraction buffer [20 mM HEPES (pH 7.9), 25% MgCl2, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF] and incubated on ice for 2h with intermittent mixing. The suspension was centrifuged, and the supernatant was collected and stored at -80°C until use.

3.11.2 Western blotting

Twenty micrograms of nuclear extracts or liver homogenates were separated by SDS PAGE on a 12% gel and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ). Membranes were incubated with primary antibodies [goat polyclonal anti-HO-1 or rabbit polyclonal anti-Nrf-2 (each 1:500; Santa cruz biotecthnology, CA), or rabbit polyclonal anti- iNOS or rabbit polyclonal anti-NF-κB (p65), or mouse monoclonal anti-actin antibodies (each 1:1,000; Abcam, Cambrige, MA)]. After washing, membranes were incubated with appropriate secondary [horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (Amersham Bioscience), or rabbit anti-goat IgG antibody (Zymed, UK)]. The immunoreactive material was visualized by enhanced chemiluminescence (ECL Western blotting kit, GE Healthcare Biosciences Corp., Piscataway, NJ). Relative band intensity was analyzed with a computer-assisted imaging densitometer system (Scion image, Scion Corporation, USA).

Table 3 Sequence of the murine primers used for real time PCR

Genes	GenBank accession NO.	Product sequence 5′→3′	Tm (°C)	Products lengths (bp)
ATF3	NM 007498.3	CCAGGTCTCTGCCTCAGAAG; AAGAGCTGAGATTCGCCATC	55	127
CAT	NM 009804.2	TTGACAGAGAGCGGATTCCT; AGCTGAGCCTGACTCTCCAG	55	179
COX2	AF 233596.1	CCATGGGTGTGAAAGGAAAT;GA AGTGCTGGGCAAAGAATG	55	131
GAPDH	DQ403054.1	AGAAGACTGTGGATGGCCCC;TGA CCTTGCCCACAGCCTT	55	110
GCL	DQ 164404.1	GTGCTGTCTGGAGAAGATGT; GTCCTCCACTGTGTTGAACT	55	165
HO-1	NM 012580.2	GCTCCTCAGGGAAGTAGAGT;GAA CTTTCAGAAGGGTCAGG	58	143
IL-1	AB 028497.1	GCCCATCTTCTGTGACTCCT; TGGAGAACACCACTTGTTGG	55	167
iNOS	DQ 355357.1	TCACCTACTTCCTGGACATC; TTTCTGAACTTCCAATCGTT	55	142
Keap1	NM 001110307.1	CGTGTAGGCGAACTCAAT; GACCCTGCAGGTCAAATA	55	190
NF-κB (p65)	DQ 020177.1	GCTCAAGATCTGCCGAGTAA; TGAGAAAAGGAGCCTCGTG	55	144
NQO1	NM 008706.5	GCAAGGTCTTCTTATTCTGG; CTGCAGACCTGGTGATATTT	53	153
Nrf2	NM010902.3	CAAGACTTGGGCCACTTA; CCTCATCACGTAACATGC	55	136
Prdx3	NM 007452.2	GGATTCCCACTTCAGTCATC; CGTAGTCTCGGGATATCTGC	55	116
Prdx6	AF 014009.1	ACTTTGAGGCCAATACCAC; TGTAAGCATTGATGTCCTTG	55	231
SOD2	NM 017051.2	CCGAGGAGAAGTACCACGAG; GCTTGATAGCCTCCAGCAAC	55	175
TGF-β	AF 046214	ACATCGACTTTCGCAAGGAC; TGGTTGTAGAGGGCAAGGAC	55	139
TNF-α	AF 046215	GACGGGCTGTACCTGGTTTA; GAGTCGGTCACCTTTCTCCA	55	237

Table 4 Sequence of amplified fragment identities to rat, mouse and human nucleotides and amino acids.

Genes	Sequence (5'→3')	cDNA fragment size	% Nucleotide (% amino acid) identities[a]		
			Rat	Mouse	Human
ATF3	CTGATGCAAATCTCAGCTCT TCCTTGACAAAGGGTGTCAG GTGTAAGCAAAATCCTCAAA CACCAGGGACCCCGGGAGG AGAGAGGCAGGGACGATGG CAGAGGCGCTCACTTCTGAG GCAGAA	124	88 (80)	86 (80)	88 (84)
CAT	TTGACAGAGAGCGGATTCCT GAGAGAGTGGTGCATGCAAA GGGAGCAGGTGCCTTTGGAT ACTTTGAGGTCACTCACGAT ATTACCAGGTACTGTAAGGC AAAGGTGTTTGAGCACATTG GAAAGAGGACCCCCATTGCC GTTCGATTCTCCACAGTCGCT GGAGAGTCAGGCTCAGCT	179	93 (98)	93 (96)	84 (94)
COX-2	CCATGGGTGTGAAAGGAAAT AAGGAGCTTCCTGATTCGAA AGAAGTTCTGGAAAAAGTTC TTCTCAGGAGAAAGTTCATC CCTGATCCCCAAGGCACGAA TATGATGTTTGCATTCTTTGC CCAGCACTTC	131	93 (97)	92 (95)	86 (81)
GAPDH	AGAAGACTGTGGATGGCCCC TCCGGGAAGCTGTGGCGTGA TGGCCGTGGGGCTGCCCAGA ACATCATCCCTGCATCCACT GGTGCTGCCAAGGCTGTGGG CAAGGTCA	108	97 (100)	98 (100)	93 (97)
GCL	GTGCTGTTCTGGAGATAGAT GTTCTTGAAACTCTGCAGGA GAAGGGGGAAAGGACAAAT CCCCAACCACCCAAGCCCTC TCGTGTAGACCGGATGTATT GGAAGTTACACTCGATTCGA AGGGACAGCTCTGCTCCAGT CCCGTAGTGTGAGTGAATCG AC	161	84 (88)	84 (88)	80 (88)

Table 4 Sequence of amplified fragment identities to rat, mouse and human nucleotides and amino acids. (Cont.)

Genes	Sequence (5'→3')	cDNA fragment size	% Nucleotide (% amino acid) identities[a]		
			Rat	Mouse	Human
HO-1	GCTCCTCCAGGGAAGTAGAG TGGGGCGTAGACTGAGGTTC TGCTTATTGCGTTCTATCTCC TCCTCCAAAGCCGTGTAGATG TGGTAACAAGGAGGCCATCA CCAGCTTAAAGCCTTCCCTGG TCACCTGACCCTTCTGAAGTT C	145	90 (94)	90 (94)	83 (94)
iNOS	TCACCTACTTCCTGGACATCA CTACCCCTCCCACCCAGCTGC AGCTCCACAAGCTGGCCCGC CTGGCCACAGATGAGGCTGA AAGGCAGAGGCTGGAGGCCC TGTGTCAGTCTTCAGAGTACA ACGATTGGAAGTTCAGAAA	176	90 (88)	87 (81)	86 (88)
IL-1β	TGGAGAACACCACTTGTTGGT TTATGTTCTGTCCGTTGAGAT GGAGAGCTTTCAGCTCACAG GGGTCAGACAGCACGAGGCA TTTCTGTTGTTCATCTCGGAG CCTGCAGTGCAGCTGTCGAAT GGGAGCATCAGCCACGATCA GCTCACCATCCCAGGAGTCA CAGAAGATGGGC	176	88 (84)	84 (80)	75 (67)
Keap1	CGTGTAGCGAACTCAATGAT GCCGCTCCATGATCCTTCGGG TGGATGCTTCGATGGACACA CTCCATCCCCTGCTCCCGAAG CCCGTTGGTGAACATGGCCTT AAAGACCGGGCTGGAGGAGG CCAGCACACACCTTGTGAGC CAATGAATTGGGCAGGCTTT GGGTGATCCCTCGGTATTTGA CCTGCGGGTC	194	84 (80)	85 (80)	83 (80)

Table 4 Sequence of amplified fragment identities to rat, mouse and human nucleotides and amino acids. (Cont.)

Genes	Sequence (5'→3')	cDNA fragment size	% Nucleotide (% amino acid) identities[a]		
			Rat	Mouse	Human
NF-κB	GCTTTGCAAACCTGGGAATACT TCATGTAACTAAGCAAAAGGT ATTTGCAACACTGGAGGCCCG GATGACAGAGGCGTGTATACG GGGCTACAATCCTGGACTTCTG GTGCATTCTGACCTTG	123	91 (95)	93 (95)	86 (92)
NQO1	GCAAGTCTTCTTATTCTGGAAA GGGCCCTTGTCGTACATGGTGG CATATGTGTAGGGCAAATTCTG CAACAAGTACTCGCTCAAACC AACCTTTCAGAATGGCGGGCA CTCCAAACCAATGCAGTGGGA ACTGAAATATCACCAGGTCTGC AG	153	90 (77)	89 (75)	82 (81)
NRF-2	CAAGACTTGGGCCACTTAAAA GACGAGAGAGAAAAACTACTC AGAGAAAAGGGAGAAAACGA CAGAAACCTCCATCTACTGAA AAGGCGGCTCAGCACCTTGTAT CTTGAAGTCTTCAGCATGTTAC GTGATGAGG	136	94 (97)	100 (100)	80 (84)
Prdx3	CGTAGTCTCGGGATATCTGCTT GGTTAAATCTGACAAGAGCGT GATGTTCATGTGCCCCAAGCCA CCATTCTTCCTTGGTGTATTGA TCCAGGCAAGATGACTGAAGT TGGAATCC	116	91 (97)	91 (94)	90 (94)
Prdx6	TGTAAGCATTGATGTCCTTGCT CCATGCAAGATGGTCCTCAAC ACTGTCTATGGAAAGAGCAAT CAACTTAACATTCCTCTTGGCA AACTCTGGCGCCAGCTTTGCAG CTCTGCCAAGCTCTGTGTGCAC ACTGGGGTAAAGTCCCGTGGG TGGGAAAAGAGAATGCCCCAT GAGTCTCCCAGGAAATCGTGT GTAAGCATTGATGTCCTTGCTC CATGCAAGATGGT	231	95 (92)	97 (92)	94 (92)

Table 4 Sequence of amplified fragment identities to rat, mouse and human nucleotides and amino acids. (Cont.)

Genes	Sequence (5'→3')	cDNA fragment size	% Nucleotide (% amino acid) identities[a]		
			Rat	Mouse	Human
SOD-2	CCGAGGAGAAGTACCACGA GGCCCTGGCCAAGGGAGATG TTACGACTCAGATTGCTCTTC AACCTGCCCTGAAAGTTCAA TGGTGGGGGACATATCAATC ACAGCATTTTCTGGACAAAC CTGAGCCCTAATGGTGGTGG AGAGCCCAAAGGAGAGTTGC TGGAGGCTATCAAGC	175	94 (97)	93 (94)	89 (100)
TGF-β	TGGTTGTAGAGGGCAAGGAC CTTACTGTACTGTGTGTCCAG GCTCCAAATGTAGGGACAGG GCCCCAGACAGAAGTTGGCG TGGTAGCCCTTGGGCTCGTG AATCCACTTCCAGCGCCATG TCCTTGCGAAAGTCGATGA	140	92 (72)	91 (88)	89 (88)
TNF-α	AGTCTGTCACCTTTCTCCAGC TGGAAGACCCCTCCCAGGTA GATGGGCTCGTACCAGGGCT TGAGCTCCTCCCCCTCAGGG GTTTCCTTGGGGCAGGGGCT CTTGATGGCGGACAGGAGGT TGACGTTATCCTCGTAAGAC ACAGCAATGCGGCTGACAGT GTGGGTGAGGAGCACGTAGC TGGGGCAGCCTTGGCCCCTG AAGAGAACCTGGGAGTAAA CCAGGTACAGCCC	236	86 (84)	87 (85)	83 (84)

[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. The GenBank accession numbers used in the sequence comparison of rat, mouse and human were as follows: ATF3, BC078903.1 (NP_037044.1), NM_007498.3 (NP_031524.2), NM_001040619.1 (AAP36398.1); CAT, NM_012520.1 (NP_036652.1), NM_009804.2 (NP_033934.2), NM_001752.3 (NP_001743.1); COX-2, AF233596.1 (AAN52933.1), NM011198.3 (AAA39918.1),

AY462100.1 (AAA57317.1); GAPDH, NW_047470.1 (ABD77186.1), XR_033853.1 (XP_001473673.1), NT_009759 (88_014556); GCL, NM_012815.2(NP_036947.1), NM_010295.1 (NP_034425.1), NM_001498.2 (NP_001489.1); HO1, NM_012580.2 (NP_036712.1), NM_010442.2 (NP_034572.1), NM_002133.2 (NP_002124.1); iNOS, AY211532.1 (NP_036743.3), NM_010927.3 (NP_035057.1), NM_000625.4 (AAC83553.1); IL-1β, NM031512.2 (NP113700.2), BC013644.1 (NP032387.1), AY888637.1 (EAW73606.1); Keap1, NM_057152.1 (EDL78313.1), NM_001110307.1 (NP_057888.1), NM_203500.1 (NP_036421.2); NF-κB, XM_3422346.4 (EDL82271.1), NM_008689.2 (EDL12142.1),NM_001165412.1 (CAB94757.1);NQO1,NM_017000.3 (AAA41988.1), NM_008706.5 (AAH04579.1), NM_000903.2 (AAP20940.1); Nrf-2, NM_031789.1 (NP_113977.1), NM_010902.3 (NP_035032.1), NM_606164.3 (NP_006155.2); Prdx3, NM_022540.1 (EDL95485.1), NM_006452.2 (NP_031478.1), NM_006793.2 (AAH08435.1); Prdx6, NM_053576.2 (NP_446028.1), NM_007453.3 (NP_031479.1), NM_004905.2 (NP_004896.1); SOD2, NM_017051.2 (CAA30928.1), NM_008160.5 (AAH86649.1), BC007865.2 (CAA68491.1); TGF-β, NM_021578.2 (EDL94931.1), NM_011577.1 (NP_035707.1), NM_000660.4 (AAP36538.1); TNF-α, AC130591.5 (EDC83548.1), BC137720.1 (BAF02299.1), DQ894849.2 (CAQ07223.1). The GenBank accession numbers are shown as nucleotide (protein) database for rat, mouse, and human, respectively.

3.12 Statistical analysis

The data are expressed as mean ± SD. Statistics significance of relative gene expression, biochemical parameters and intensity of bands of protein expression levels were determined using one-way ANOVA followed by Turkey's multiple comparison tests. Student *t*-test was used to compare the number of inflammatory cells and Nrf2 positive index. Non-parametric Mann-Whitney *U* test was used to determine the number of HO-1 positive index. Statistical analyses were performed using SPSS version 15. *P* values less than 0.05 were consider statistically significant.