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Curcumin protects the rat liver from CCl₄-caused injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation

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ABBREVIATIONS

CCl₄, Carbon tetrachloride; **DMEM**, Dulbecco's modified Eagle's medium; **ECM**, extracellular matrix; **EGFR**, epidermal growth factor receptor; **FBS**, fetal bovine serum; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **GSH**, Glutathione; **HSC**, hepatic stellate cells; **IFN- γ** , interferon-gamma; **IL-6**, interleukin-6; **LPO**, lipid peroxidation; **PDGF β R**, platelet-derived growth factor-beta receptor; **PPAR γ** , peroxisome proliferator-activated receptor-gamma; **TGF- β R**, transforming growth factor-beta receptor; **TNF- α** , tumor necrosis factor-alpha.

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ABSTRACT

We previously demonstrated that curcumin, a polyphenolic antioxidant purified from turmeric, up-regulated PPAR γ gene expression and stimulated its signaling, leading to the inhibition of activation of hepatic stellate cells (HSC) *in vitro*. The current study evaluates the *in vivo* role of curcumin in protecting the liver against injury and fibrogenesis caused by carbon tetrachloride (CCl $_4$) in rats and further explores the underlying mechanisms. We hypothesize that curcumin might protect the liver from CCl $_4$ -caused injury and fibrogenesis by attenuating oxidative stress, suppressing inflammation and inhibiting activation of HSC. This report demonstrates that curcumin significantly protects the liver from injury by reducing the activities of serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, and by improving the histological architecture of the liver. In addition, curcumin attenuates oxidative stress by increasing the content of hepatic glutathione, leading to the reduction in the level of lipid hydroperoxide. Curcumin dramatically suppresses inflammation by reducing levels of inflammatory cytokines, including IFN- γ , TNF- α and IL-6. Furthermore, curcumin inhibits HSC activation by elevating the level of PPAR γ and reducing the abundance of PDGF, TGF- β , their receptors and type I collagen. This study demonstrates that curcumin protects the rat liver from CCl $_4$ -caused injury and fibrogenesis by suppressing hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSC activation. These results confirm and extend our prior *in vitro* observations and provide novel insights into the mechanisms of curcumin in the protection of the liver. Our results suggest that curcumin might be a therapeutic anti-fibrotic agent for the treatment of hepatic fibrosis.

INTRODUCTION

Hepatic fibrosis is the wound response to chronic hepatic injury, including alcohol abuse, viral infection, cholestasis, etc. It is characterized by excessive production and deposition of extracellular matrix (ECM) molecules (Bataller and Brenner, 2005; Friedman, 2003). Research over the past two decades has established that hepatic stellate cells (HSC) are the primary ECM-producing cell type during hepatic fibrogenesis (Bataller and Brenner, 2005; Friedman, 2003). HSC activation, characterized by enhanced cell growth and over-production of ECM, is triggered by the release of mitogenic platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) from activated HSC and fibrogenic transforming growth factor-beta1 (TGF- β 1) mostly from Kupffer cells (Bataller and Brenner, 2005; Friedman, 2003). This process is coupled with the sequential up-expression of PDGF-beta receptor (PDGF- β R) (Wong et al., 1994), type I & II receptors for TGF- β (T β -RI & T β -RII) (Friedman et al., 1994) and EGF receptor (EGFR) (Komuves et al., 2000). In addition, HSC activation coincides with a dramatic reduction in the peroxisome proliferator-activated receptor-gamma (PPAR γ) (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000). Inflammation is a key event in the stimulation of HSC activation and hepatic fibrosis, which are induced by oxidative stress (Greenwel et al., 2000; Lee et al., 1995; Tsukamoto et al., 1995).

We previously reported that the polyphenolic antioxidant curcumin, the yellow pigment in curry from turmeric, inhibited activation of HSC *in vitro* by reducing cell proliferation, inducing apoptosis and suppressing ECM gene expression (Xu et al., 2003). Further studies demonstrated that curcumin induced gene expression of PPAR γ in activated HSC *in vitro*, leading to the activation of PPAR γ signaling. The latter is a

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prerequisite for curcumin to inhibit HSC activation (Zheng and Chen, 2004; Zheng and Chen, 2006; Zhou et al., 2007). The current study evaluates the *in vivo* role of curcumin in the protection of the liver from injury and fibrogenesis caused by carbon tetrachloride (CCl₄) in a rat model and further explores the underlying mechanisms. We hypothesize that curcumin might protect the liver from CCl₄-induced injury and fibrogenesis by attenuating oxidative stress, suppressing inflammation and inhibiting activation of HSC. Results in this study support our hypothesis, extend our prior *in vitro* observations and provide novel insights into the mechanisms of curcumin in the protection of the liver.

MATERIALS AND METHODS

Establishment of a rat model with hepatic injury and fibrogenesis caused by

CCl₄: The rat model was established using the method originally described by Proctor and Chatamra (Proctor and Chatamra, 1982) and used by many others (Kobayashi et al., 2000; Perez Tamayo, 1983; Rivera et al., 2001), with minor modifications. Thirty male Sprague-Dawley rats (250-300g) were randomly divided into 5 groups (6 rats /group). The Group #1 was the Vehicle Control administered with no CCl₄, nor curcumin, only intraperitoneally (*i. p.*) injected with the vehicle olive oil. The Group #2 was the Curcumin Control injected with olive oil like the Group #1, without CCl₄, but orally given curcumin. The Group #3 was the CCl₄ Group *i. p.* injected with CCl₄, without curcumin treatment. The Group #4 was a treatment group injected with CCl₄ and treated with curcumin at 200 mg/kg. The Group #5 was another treatment group injected with CCl₄ and treated with curcumin at 400 mg/kg. All rats were fed with chow diet and kept at 21°C-25°C under 12-hour dark/light cycles. Rats in Group # 3, 4 & 5 were *i. p.* injected with a mixture of CCl₄ (0.1ml/100g body weight) and olive oil (1:1 v/v) every other day for 8 weeks. Curcumin (200mg/kg, or 400mg/kg body weight) was suspended in sterile PBS and given once daily by gavage. The control animals in the Group # 1 & 2 were similarly handled, including *i.p.* injection with the same

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volume of olive oil and oral administration of the same volume of PBS with or without curcumin. Forty-eight hours after the last CCl₄ injection, rats were sacrificed after being anesthetized by *i.p.* pentobarbital (50mg/kg). A small portion of the liver was removed for immuno-histochemical studies by fixation with formalin (10%) and subsequent embedment with paraffin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of total RNA, hepatic protein extracts, or others.

Analyses of the pathological indexes for hepatocytic death and hepatic injury:

Blood was collected from each rat by heart puncture when sacrificed. After coagulation, sera were collected and stored at -20°C for further analyses. Activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in fresh sera (1ml/sample) were analyzed by the Chemical Lab in the Department of Pathology, Louisiana State University Health Science Center in Shreveport.

Enzyme-linked immuno-Sorbent Assays (ELISA): Levels of hepatic or serum IFN- γ , TNF- α , IL-6, PDGF and TGF- β in rats were determined by using a corresponding ELISA kit purchased from BD Biosciences (San Jose, CA) according to the protocol provided by the manufacturer. Briefly, microplates were coated with 100 μ l/well of capture antibody and incubated overnight at 4°C. After washes, the plates were blocked with Assay Diluent at room temperature (RT) for 1 hour. One hundred microliter of a serum sample, or a liver extract in PBS supplemented with protease inhibitors was added to each well of the plate, followed by incubation for 2 hours at RT. Working Detector (100 μ l) was loaded into each well and incubated for an additional 1 hour at RT prior to the addition of Substrate Solution (100 μ l). The reaction was stopped by adding Stop Solution (50 μ l). The absorbance was read at 450nm with reference wavelength at 570nm using a 96-well plate spectrometer (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). Calculation of the

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concentrations of the cytokines was performed in a log-log linear regression according to the instructions in the protocol.

Determination of the content of hepatic hydroxyproline: This experiment was performed using a colorimetric method described by Bergman and Loxley with modifications (Bergman and Loxley, 1970). In brief, three small pieces of liver tissues randomly excised from the liver of every rat in the rat model were hydrolyzed in 6N HCl at 110°C for 24hr and subsequently neutralized with NaOH. Isopropanol in citrate-acetate-buffered Chloramine T was added to aliquots of the hydrolysate, followed by the addition of Ehrlich reagent. The chemical reaction occurred in dark for 25 min at 60°C. After centrifugation, absorbance of the supernatant of each sample was read at 558 nm using a 96-well plate spectrometer (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). Trans-hydroxyproline was used as the standard for quantification. Results were expressed as μmol hydroxyproline/ gram of hepatic tissue.

GSH assays: Levels of hepatic GSH and GSSG were determined using the enzyme immune assay kit GSH-400® (Cayman, Ann Arbor, MI), following the protocol provided by the manufacturer. Briefly, small pieces of the liver tissue collected from the rat model were homogenized and sonicated in 0.5 ml of cold buffer (50mM MES, pH 7.0 containing 1mM EDTA) and centrifuged at 10,000g for 15 min at 4°C. The supernatant was collected and deproteinated using the reagent in the kit. A sample (50 μl /well) and the Assay Cocktail (150 μl /well) were mixed and incubated in dark on a plate shaker. The absorbance was measured at 404nm after 25-minute incubation. A GSSG standard was generated according the instruction. The concentration of total GSH was calculated according to the equation in the protocol.

Analyses of lipid peroxidation (LPO): The LPO assays were carried out using a Lipid Hydroperoxide Assay Kit purchased from Cayman Chemical (Ann Arbor, MI). A piece

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of liver tissue was collected from each rat of the rat model. Immediately after sonication in PBS, lipid hydroperoxides were extracted from the samples into chloroform using the extraction buffer provided by the manufacturer. Chromogenic reaction was carried out at RT for 5 min. The absorbance was read at 500nm using a 96-well plate spectrometer (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). 13-HpODE (13-hydroperoxy-octadecadienoic acid) was used as the standard. Cellular lipid hydroperoxide was calculated and expressed as nanomoles/mg total protein.

RNA isolation and real-time PCR: Total RNA was extracted from frozen liver tissues using TRI-reagent according to the protocol provided by the manufacturer (Sigma, St. Louis, MO). Real-time PCR was performed as we previously described (Chen et al., 2002). Total RNA (1µg) was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using reverse transcription system (Promega, Madison, WI). Reverse transcription was carried out as follows: 42°C for 30min, 95°C for 5min, 4°C for 5min (one cycle). Real-time PCR was performed in a 25µl of reaction solution containing 12.5µl of 2X iQ™ SYBR Green Supermix (Bio-Rad), 300nM of primers and cDNAs. The cycles for PCR were as follows: 95°C for 7min, 40 cycles of 95°C 20s, 54°C 30s, 72°C 30s. Melting curves were determined by heat-denaturing PCR products over a 35°C temperature gradient at 0.2°C/s from 60 to 95°C. mRNA fold change relative to GAPDH was calculated as suggested by Schmittgen et al. (Schmittgen et al., 2000). GAPDH was used as an internal control. The primers were the following: **PDGF-βR:** (F), 5'-CTG CCA CAG CAT GAT GAG GAT TGA T-3', (R), 5'-GCC AGG ATG GCT GAG ATC ACC AC-3'; **EGFR:** (F), 5'-TGC ACC ATC GAC GTC TAC AT-3', (R), 5'-AAC TTT GGG CGG CTA TCA G-3'; **Tβ-R1:** (F), 5'-ATC CAT GAA GAC TAT CAG TTG CCT-3', (R), 5'-CAT TTT GAT GCC TTC CTG TTG GCT-3'; **Tβ-R2:** (F) 5'-TGT GCT CCT GTA ACA CAG AG-3', (R) 5'-GAT CTT GAC AGC CAC GGT CT-3'; **CTGF:** (F) 5'-TGT GTG ATG AGC

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CCA AGG AC-3', (R) 5'-AGT TGG CTC GCA TCA TAG TTG-3'; **α 1(I)-collagen**: (F) 5'-CCT CAA GGG CTC CAA CGA G-3', (R) 5'-TCA ATC ACT GTC TTG CCC CA-3; **Fibronectin**: (F) 5'-TGT CAC CCA CCA CCT TGA-3' (R) 5'-CTG ATT GTT CTT CAG TGC GA-3'; **α -SMA**: (F) 5'-CCG ACC GAA TGC AGA AGG A-3', (R) 5'-ACA GAG TAT TTG CGC TCC GGA-3'; **PPAR- γ** : 5'-ATT CTG GCC CAC CAA CTT CGG-3', (R), 5'-TGG AAG CCT GAT GCT TTA TCC CCA-3'; **GCLc** (F), 5'-GTC TTC AGG TGA CAT TCC AAG C-3', (R), 5'-TGT TCT TCA GGG GCT CCA GTC-3'; **GCLm** (F), 5'-CTG CTA AAC TGT TCA TTG TAG G-3', (R), 5'-CTA TGG GTT TTA CCT GTG-3'; **GAPDH**: (F) 5'-GGC AAA TTC AAC GGC ACA GT-3', (R), 5'-AGA TGG TGA TGG GCT TCC C-3'. mRNA levels were expressed as fold changes after normalization with GAPDH.

Western blotting analyses: Liver extracts were prepared from pieces of liver tissues excised from every rat in the rat model using ice-cold RIPA lysis buffer containing NaCl (150mM), Tris (50mM), SDS (0.1%), NP-40 (1%) and deoxycholate (0.5%) supplemented with protease inhibitors. Protein concentrations were determined using the BCA™ Protein Assay Kit according to the protocol provided by the manufacturer (Pierce, Rockford, IL). Forty micrograms of total proteins were subjected to SDS-PAGE (10%). GCLc or GCLm proteins were respectively detected by using primary antibodies against GCLc or GCLm and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). β -actin was probed as an internal control. Protein bands were visualized by using chemiluminescence reagent (Amersham).

Sirius red collagen staining: Rat liver tissues were fixed with buffered neutral formalin (10%) and embedded in paraffin. Thin sections (5 μ m) were deparaffinized and stained with picro-sirius red for 1 hour at RT. After washes, sections on the slides were dehydrated in ethanol (100%) and in xylene and mounted in Permount. Representative views of liver sections are shown.

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Immuno-fluorescence staining: After deparaffin, thin sections (5 μ m) of the liver tissues were blocked with BSA (1%) and then incubated with the primary antibodies overnight at 4°C. The primary antibodies used in this study were rabbit antibodies against PDGF- β R (1:200), EGFR (1:200), T β -RII (1:200), T β -RI (1:200) (Santa Cruz, CA), or PPAR- γ (1:50, Abcam Inc. Cambridge, MA), and mouse monoclonal antibodies against α -SMA (1:100, Sigma). After three washes with PBS, sections on slides were incubated with goat anti-rabbit rhodamine conjugated secondary antibodies and donkey anti-mouse Alexor Fluor 488 conjugated secondary antibodies (Molecular Probes, Invitrogen Corporation, Carlsbad, CA) at RT for 1 hour. Sections incubated with secondary antibodies alone were used as negative controls. Sections were viewed in a single plane under a Bio-Rad MRC 1024 laser confocal microscope. Images were captured using Laser Sharp software (Bio-Rad, Richmond, CA). Representative views of liver sections are shown.

Statistical analyses: Differences between means were evaluated using an unpaired two-sided Student's t-test ($p < 0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions with control were analyzed by ANOVA with the Dunnett's test for post hoc analysis.

RESULTS

Curcumin protects the liver against CCl₄-induced injury and suppresses hepatic fibrogenesis in the rat model

CCl₄ causes hepatic injury, including hepatocytic necrosis, steatosis, and inflammation (Perez Tamayo, 1983). Low-dose and long-term administration of CCl₄ induces hepatic fibrogenesis, which largely imitates hepatic fibrosis in human diseases (Perez Tamayo, 1983). A CCl₄-rat model was established as described in "MATERIALS AND METHODS" to evaluate the effect of curcumin on protecting the liver from injury and

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fibrogenesis. The process lasted for eight weeks. The liver rapidly recovers from CCl₄-caused injury (Johnson et al., 1992; Shi et al., 1998). Rats were, therefore, scarified within 48 hr after the last CCl₄ injection, which provided a better chance to evaluate the CCl₄-caused hepatic injury and to assess the effect of curcumin on protection of the liver (Johnson et al., 1992; Shi et al., 1998). The effects of curcumin on the protection of the liver from injury and fibrogenesis were initially evaluated by histological analyses. Representative views of liver sections are shown in Fig. 1. As shown in Fig. 1A stained with hematoxylin-eosin (H & E), compared to livers in the controls (Group #1 and #2), CCl₄ caused prominent hepatic steatosis, necrosis and formation of regenerative nodules and fibrotic septa between the nodules (Group #3). Oral administration of curcumin daily for 8 weeks significantly improved the state of steatosis with a significant reduction in the number of macro-, and micro-vesicular steatosis, and apparently suppressed hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa (Group #4 and #5, respectively).

To assess the impact of curcumin on hepatic fibrogenesis caused by CCl₄, liver sections were stained with picro-Sirius red for detecting the deposition of collagens. Compared to those from the control groups (Fig. 1B, #1 and #2), liver sections from rats injected with CCl₄ showed prominent red staining in the fibrotic septa between nodules (Fig. 1B, #3), suggesting a high level of collagen deposition. Curcumin treatment remarkably reduced the size stained with Sirius red in the liver (Fig. 1B, # 4 & #5).

Alpha-smooth muscle actin (α -SMA) is the unique marker for activated HSC. To evaluate the effect of curcumin on HSC activation *in vivo*, α -SMA-positive cells were examined in liver tissues from the CCl₄-rat model using immuno-fluorescence staining. Compared to that in the control groups (Fig. 1C, #1 & 2), the number of the α -SMA-positive cells around the fibrotic septa was significantly increased by CCl₄ (Fig. 1C, #3), confirming

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that CCl₄ stimulated the activation of HSC in the rat model. The number of the α -SMA-positive cells was apparently reduced by oral administration of curcumin at both doses of 200 and 400 mg/kg b. w. (Fig. 1C, #4 & #5), suggesting that curcumin inhibited HSC activation in the rat model.

Hydroxyproline is an amino acid found almost exclusively in collagens. Determination of the content of hydroxyproline in the liver tissue is regarded as a good method to quantify fibrosis and to evaluate the effectiveness of new potentially anti-fibrotic agents (Kusunose et al., 2002; Yoshiji et al., 2002). The efficacy of the treatment with curcumin on the protection of the liver from fibrogenesis was further evaluated by using a quantitative method to determine the content of hepatic hydroxyproline in the rat model. As shown in Table 1, compared to the vehicle control (Group #1), the content of hepatic hydroxyproline was significantly higher in rats injected with CCl₄ (Group #3). The level of hepatic hydroxyproline was significantly reduced in the rats treated with curcumin at 200 or 400 mg/kg b. w. (Group #4 or #5). However, there was no dose-dependently significant difference between the two groups (Group #4 and #5), suggesting that over-dose of curcumin might be used for the treatment. These results confirmed that curcumin suppressed hepatic fibrogenesis in the rat model.

Additional biochemical analyses of serum enzymes were performed to verify the role of curcumin in the protection of the liver from injury. As shown in Table 2, compared to those in the control groups #1 or #2, the activities of serum alkaline phosphatase (ALK), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were significant higher in rats injected with CCl₄ (Group #3). The levels of serum ALK, ALT and AST were significantly reduced by administration of curcumin at 200mg/kg or 400mg/kg (Group #4 or #5). However, no dose-dependent difference was observed between the two groups (Group #4 and #5). Taken together, these results demonstrated that curcumin not only

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protected the liver against CCl₄-induced injury, but also inhibited HSC activation and reduced hepatic fibrogenesis caused by chronic CCl₄ intoxication.

Curcumin suppresses inflammation in the CCl₄-rat model

Inflammation is commonly associated with hepatic fibrogenesis during chronic liver diseases (Marra, 2002). To begin to explore the mechanisms underlying the protective effects of curcumin, we proposed that curcumin might protect the liver against CCl₄-induced injury by suppressing inflammation in the liver. A piece of liver tissue and a blood sample were collected from every rat in each group. The levels of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF- α), Interferon-gamma (IFN- γ) and interleukine-6 (IL-6) were determined in the liver and in serum. As shown in Fig. 2, compared with those in the control groups #1 and #2, the levels of the three pro-inflammatory cytokines in the group #3 were significantly elevated by CCl₄ in both the liver and serum. Curcumin treatment significantly reduced the levels of TNF- α , IFN- γ and IL-6 in the group #4 and #5. There was, however, no dose-dependently significant difference between the two groups (Group #4 and #5). These results indicated that curcumin suppressed inflammation caused by CCl₄, which might lead to the protection of the liver from injury.

Curcumin reduces the levels of PDGF and TGF- β in the CCl₄-rat model

PDGF is the most potent growth factor in stimulating HSC proliferation (Bachem et al., 1989a; Pinzani et al., 1989), while TGF- β is the major pro-fibrogenic factor during hepatic fibrogenesis (Gressner et al., 2002). To elucidate the underlying mechanisms of curcumin in the suppression of hepatic fibrogenesis in the rat model, it was plausible to examine the effect of curcumin on the production of PDGF and TGF- β in the rat model. A piece of liver

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tissue and a blood sample were collected from every rat in each group, and the levels of PDGF and TGF- β in the liver and in serum were analyzed by ELISA. As shown in Fig. 3, compared with those in the vehicle control (Group #1), the levels of PDGF and TGF- β in the liver (Fig. 3A) and in serum (Fig. 3B) were dramatically increased in Group #3 injected with CCl₄. The levels of PDGF and TGF- β in the liver (Fig. 3A) and serum (Fig. 3B) were significantly reduced by curcumin treatment at both 200 and 400mg/kg (Group #4 & 5). Collectively, these data indicated that curcumin significantly reduced the levels of PDGF and TGF- β in the rat model, which might result in the inhibition of HSC activation stimulated by CCl₄.

Curcumin abrogates the effect of CCl₄ on altering the steady state mRNA levels of genes relevant to HSC activation in the liver in the CCl₄-rat model

As indicated earlier, HSC activation is coupled with the sequential up-expression of PDGF- β R, EGFR, T β -RI and T β -RII. In addition, HSC activation coincides with a dramatic reduction in PPAR γ . We previously reported that curcumin inhibited HSC activation *in vitro* demonstrated by suppressing expression of genes relevant to cell growth and fibrogenesis (Xu et al., 2003; Zheng and Chen, 2004). In addition, we observed that curcumin induced expression of endogenous PPAR γ gene and stimulated PPAR γ activity (Xu et al., 2003), which was required for curcumin to inhibited HSC activation *in vitro* (Xu et al., 2003; Zheng and Chen, 2004). To verify these effects of curcumin on the regulation of expression of genes relevant to HSC activation in the rat model, total RNA was extracted from the liver of each rat in the rat model. The steady state levels of mRNA of genes relevant to HSC activation in the liver were evaluated using real-time PCR. As shown in Fig. 4A, compared with that in the vehicle control (Group #1), the mRNA level of PPAR γ was markedly

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reduced by 68% in the rats administered with CCl₄ (Group #3). The level of PPAR_γ mRNA was significantly elevated by simultaneous treatment with curcumin by approximately 480% in Group #4. There was no significant difference between groups treated with curcumin at 200 or 400 mg/kg (Group #4 and #5). It was of interest to observe that curcumin alone increased the mRNA level of PPAR_γ, as well (Group #2). In great contrast, the mRNA levels of α-SMA, the marker of activated HSC, and α1(I) collagen were significantly increased by approximately 3.4 and 5.3 fold, respectively, in the CCl₄ group (#3 in Fig. 4B). Curcumin treatment apparently abrogated the stimulatory effect of CCl₄ and reduced the abundance of mRNA of α-SMA and α1(I) collagen by approximately 48% and 47%, respectively (Group # 4 in Fig. 4B). These results suggested that curcumin might inhibit HSC activation and suppress fibrogenesis in the rat model. Additional experiments demonstrated that, compared to the controls (Group #1 & 2), CCl₄ significantly induced expression of genes relevant to cell proliferation, including PDGF-βR and EGFR (Group #3 in Fig. 4A), and genes relevant to fibrogenesis, including Tβ-RI & Tβ-RII and fibronectin (FN) (Group #3 in Fig. 4B). Curcumin markedly eliminated the role of CCl₄ in the rat model by reducing the mRNA levels of these genes (Group #4 & 5 in Fig. 4A & B). Curcumin showed no dose-dependent effect on regulation of expression of the genes, suggesting that over-dose of curcumin might be used for the treatment. Taken together, these results indicated that curcumin abrogated the effect of CCl₄ on altering the steady state levels of mRNA of genes relevant to HSC activation in the liver in the CCl₄-rat model.

Curcumin eliminates the impact of CCl₄ on regulating the abundance of proteins relevant to HSC activation in the liver in the CCl₄-rat model

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Immuno-histochemical (IHC) experiments were performed to further evaluate the impact of curcumin on regulating the expression of genes relevant to HSC growth. Liver sections from each group were doubly immuno-labeled with antibodies against PDGF- β R and against α -SMA, the marker of activated HSC. As shown in Fig 5A, as expected, few cells in the liver sections from the normal control groups were recognized by antibodies against PDGF- β R or against α -SMA (Fig. 5A, #1 and #2), suggesting few activated HSC in the normal livers in the control rats. Administration of CCl₄ caused a significant increase in the number of cells recognized by the antibodies against PDGF- β R (the upper view with red fluorescence in Fig. 5A, #3), or α -SMA (the middle view with green fluorescence in Fig. 5A, #3). Merging the images demonstrated that these labeled cells by the two different antibodies were the same cells (the lower view in Fig. 5A, #3), suggesting the co-localization of PDGF- β R and α -SMA in these cells, *i.e.* activated HSC. Curcumin treatment significantly reduced the number of cells labeled with PDGF- β R and α -SMA antibodies (Fig. 5A, #4 & 5), suggesting that curcumin might suppress HSC activation by inhibiting cell growth of HSC in the rat model. The similar pattern was observed when the liver sections from the rat model were doubly labeled with antibodies against EGFR, another important mitogenic protein relevant to cell proliferation, and antibodies against α -SMA (Fig. 5B).

Additional IHC was performed to verify the effect of curcumin on regulating expression of genes relevant to fibrogenesis, including T β -RI and T β -RII. As shown in Group #1 and 2 in Fig. 6, the expression of T β -RI and T β -RII is hardly detectable in the normal rat liver. Curcumin has no apparent impact on the abundance of T β -RI and T β -RII (Group #2, Fig. 6). Interestingly, administration of CCl₄ significantly increases the number of cells recognized by the antibodies against T β -RI, or T β -RII, (the upper views with red fluorescence in Fig. 6A, #3 or in Fig. 6B, #3, respectively), or α -SMA (the middle views

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with green fluorescence in Fig. 6A & B, #3). Curcumin significantly reduced the number of the positively labeled cells (Group #4 & #5 in Fig. 6). These results collectively confirmed the prior observations in Fig. 4 and indicated that curcumin suppressed expression of genes relevant to fibrogenesis, leading to the inhibition of HSC activation in the rat model.

Prior studies have demonstrated that the elevation in the expression of α -SMA, the marker of activated HSC, is coupled with the reduction in the expression of PPAR γ in activated HSC (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000; Xu et al., 2003). To verify our prior *in vitro* observation that curcumin induced gene expression of PPAR γ in HSC, IHC of the liver sections was conducted using antibodies against PPAR γ and α -SMA. As shown in Fig. 7, there were numbers of PPAR γ positively stained cells, which were negatively labeled with α -SMA antibodies, in liver sections from Group #1 and #2. Along with the increase in the number of α -SMA positively labeled cells in the liver from rats injected with CCl $_4$ (Group #3), the number of cells labeled with PPAR γ was remarkably reduced. Simultaneous treatment with curcumin apparently eliminated these roles of CCl $_4$ in the alteration of the number of PPAR γ -positive cells and α -SMA-positive cells in the liver (Group #4 and #5). Taken together, these results demonstrated that curcumin inhibited expression of genes relevant to HSC activation and diminished the role of CCl $_4$ in the suppression of expression of PPAR γ gene in HSC, which verified our prior *in vitro* observations.

Curcumin significantly attenuates oxidative stress in the liver caused by CCl $_4$

Oxidative stress resulting from the metabolism of CCl $_4$ in the liver plays a critical role in damaging the liver and promoting hepatic fibrogenesis. Glutathione (γ -glutamyl-cysteinyl-glycine; GSH) is the predominant low molecular weight thiol and the most

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important non-enzyme antioxidant in mammalian cells (Wu et al., 2004). GSH effectively protects cells against oxidative stress-caused damage by scavenging free radicals, removing hydrogen peroxide (H_2O_2) and suppressing lipid peroxidation (LPO) (Blair, 2006). We recently demonstrated that curcumin inhibited HSC activation *in vitro* through attenuating oxidative stress via de novo synthesis of glutathione (Zheng and Chen, 2007). To evaluate the impact of curcumin on attenuating oxidative stress *in vivo*, the level of hepatic GSH and the ratio of reduced GSH (*i.e.* GSH) verse oxidized GSH (*i.e.* GSSG) were determined. The GSH/GSSG ratio is often regarded as a sensitive indicator of oxidant stress (Fridovich, 1978; Jones, 2002). As shown in Fig. 8A and B, compared with that in the Vehicle Control (Group #1), the level of total GSH, as well as the ratio of GSH/GSSG, were significantly reduced by CCl_4 by 59% and 42%, respectively (Group #3). Oral administration of curcumin apparently eliminated the inhibitory effect of CCl_4 by increasing the level of total hepatic GSH by 88% and 75%, respectively (Group #4 & 5, Fig. 8A), and by elevating the ratio of hepatic GSH/GSSG by 52% and 61% (Group #4 & 5, Fig. 8B). Furthermore, we did additional experiments to verify the effect of curcumin on the attenuation of oxidative stress in the liver of the rat model. As shown in Fig. 8C, CCl_4 increased the level of lipid hydroperoxide, a major product of LPO, by 62% (Group #3, Fig. 8C) compared with that in the normal control (Group #1). Oral administration of curcumin significantly diminished the CCl_4 -elevated level of lipid hydroperoxide in the rat model by 40% and 42%, respectively (Group #4 & 5, Fig. 8C). These data collectively suggested that curcumin might protect the liver against CCl_4 -caused injury by attenuating oxidative stress.

Curcumin apparently eliminates the inhibitory effect of CCl_4 on the GCL activity by inducing gene expression of GCL in the rat model

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Additional experiments were conducted to elucidate the underlying mechanisms of curcumin in the elevation of the level of hepatic GSH and the attenuation of oxidative stress. GSH is sequentially synthesized from glutamate, cysteine and glycine, which is mainly controlled by the rate-limiting enzyme glutamate-cysteine ligase (GCL). GCL is composed of two subunits, *i.e.* the heavy catalytic subunit GCLc (73kDa) and the light regulatory subunit GCLm (31kDa). We postulated that oral administration of curcumin might eliminate the inhibitory effect of CCl₄ on the GCL activity in the rat model by inducing expression of GCL subunit genes, leading to the elevation of the GSH content and the attenuation of oxidative stress. To test the postulation, the GCL activity was analyzed in liver tissues collected from rats in each group. As shown in Fig. 9A, CCl₄ significantly reduced the GCL activity in the rat model (Group #3). Oral administration of curcumin apparently eliminated the inhibitory effect of curcumin on the activity of GCL (Group # 4 & 5, Fig. 9A). Further experiments revealed that curcumin significantly enhanced the levels of the transcript and protein of GCL subunits, GCLc and GCLm in the rat model, demonstrated by real-time PCR (Group # 4 & 5, Fig. 9B) and Western blotting analyses (Group # 4 & 5, Fig. 9C). These results collectively demonstrated that curcumin apparently eliminated the inhibitory effect of CCl₄ on the GCL activity by inducing gene expression of GCL in the rat model.

DISCUSSION

Hepatic fibrosis caused by CCl₄ has been extensively used in experimental models in rats. Hepatic responses in rats to chronic CCl₄ stimulation is shown to be superficially similar to human cirrhosis (Perez Tamayo, 1983). CCl₄ metabolism in the liver results in the stimulation of lipid peroxidation and the production of free radical (Basu, 2003), which causes necrosis of hepatocytes, induces inflammation and further promotes progression of

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hepatic fibrogenesis. The number of 6 rats in each group was chosen as the sample size in this report. A difference within two groups with a *p-value* of less than 5% was considered to be significant (positive). This probability was computed under the assumption that the treatment difference or strength of association equaled the minimal detectable difference. In the present study, we demonstrated that curcumin significantly reduced the pathological indexes for hepatocytic death and hepatic injury caused by CCl₄ in the rat model. In addition, curcumin attenuated hepatic oxidative stress and dramatically suppressed inflammation initiated by CCl₄. Furthermore, curcumin apparently reduced the deposition of collagens and altered the expression pattern of genes relevant to HSC activation in this rat model. Taken together, our results demonstrated that curcumin protected the rat liver from CCl₄-caused injury and fibrogenesis by suppressing hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSC activation. However, it is noteworthy that, except for some of our histological experiments, most of our biochemical and molecular assays observed no significant difference between administration of curcumin at 200 and 400 mg/kg in the protection of the liver from injury, the attenuation of oxidative stress, the suppression of inflammation and the regulation of the gene expression. We assumed that over-dose of curcumin might be used in our experiments compared to curcumin at 100mg/kg in some other reports (Sreepriya and Bali, 2006). An even lower dose of curcumin at 40mg/kg appeared to be effective in the treatment of mice with cystic fibrosis (Egan et al., 2004). Additional experiments are ongoing using lower doses of curcumin in the rat model.

In this report, traditional methods of assessing hepatic fibrosis, such as histopathological analysis and hydroxyproline measurements, were performed to determine the effectiveness of curcumin on the protection of the liver from CCl₄-induced fibrogenesis. Results from histopathological analyses demonstrated that curcumin

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significantly reduced the size stained with Sirius red in the liver. In addition, results from determination of the level of hepatic hydroxyproline indicated that the CCl₄-elevated levels of hepatic hydroxyproline were significantly reduced by oral administration of curcumin, which confirmed the histological observations in Fig. 1. Both results suggested that curcumin protected the liver from fibrogenesis caused by CCl₄ in the rat model. However, it is noteworthy that the haphazard distribution of fibrous tissue within the liver, such as in septa, bands or expanded portal spaces, could interfere with measurement of the level of hepatic hydroxyproline and mislead the final conclusion. A recent study indicated that hydroxyproline ratio in a normal liver kept a fair degree of reproducibility (Gomes et al., 2006). However, in livers with fibrosis, the levels of hydroxyproline varied significantly among samples excised from the same liver, suggesting that the level of hydroxyproline in the liver might have limited value in quantifying the extent of fibrosis (Gomes et al., 2006).

Inflammation is commonly associated with hepatic fibrogenesis during chronic liver diseases (Marra, 2002). CCl₄ is metabolized in the liver by cytochrome p450 into free radical CCl₃· (Burk et al., 1984). The free radical attacks hepatocytes and causes necrosis of parenchymal cells, which promotes inflammatory responses in the liver (Perez Tamayo, 1983). Pro-inflammatory TNF-α, IFN-γ and IL-6 are major players in hepatic inflammation. Concentrations of serum TNF-α and IL-6 are remarkably increased in patients with alcoholic cirrhosis (Byl et al., 1993; Diez Ruiz et al., 1993). TNF-α stimulates the development of hepatic fibrosis (Yin et al., 1999). IL-6 induces hepatic inflammation and collagen synthesis *in vivo* (Choi et al., 1994). IL-6 produced by activated HSC facilitates the production of ECM, including type I collagen, leading to hepatic fibrosis (Natsume et al., 1999). We reported in the present study that curcumin significantly reduced the levels of TNF-α and IL-6 in the liver and in serum in the CCl₄-rat model. Our observations are consistent with and supported by prior observations. Fibrotic changes were less evident in

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IL-6 deficient mice injected with CCl₄ (Natsume et al., 1999). Curcumin ameliorated the alcoholic and non-alcoholic experimental pancreatitis and reduced mRNA levels of IL-6, TNF- α and iNO synthase (Gukovsky et al., 2003). IFN- γ plays a crucial role in modulating immune responses. Prior studies reported the inhibitory role of IFN- γ in HSC proliferation *in vitro* (Rockey et al., 1992; Shen et al., 2002). However, our study showed that the level of IFN- γ was positively correlated with serologic markers of hepatic injury and fibrogenesis. Prior clinical reports also indicated that IFN- γ was positively associated with elevated levels of ALT in chronic hepatitis B patients (Hyodo et al., 2003; Lee et al., 1999). The role of the elevated level of IFN- γ in the development of hepatic injury and fibrogenesis remains unclear. It was postulated that the elevation in the level of IFN- γ might be advantageous in the control of hepatic viral infection (Wang et al., 2007). It is, therefore, plausible to assume that in addition to its role in the induction of inflammation, the elevation of the level of IFN- γ in the rat model might be an advantageous response for the animals to protect the liver from CCl₄-caused injury and to suppress HSC proliferation. Additional experiments are necessary to determine the role and mechanisms of INF- γ in hepatic fibrogenesis.

Oxidative stress is closely associated with hepatic fibrosis (Poli and Parola, 1997). Lipid peroxidation and necrosis are significantly suppressed in the liver of animals supplemented with antioxidants such as flavonoid, silymarin or vitamin E (Parola and Robino, 2001). We recently reported that curcumin elevated the level of cellular GSH and induced *de novo* synthesis of GSH in HSC by stimulating the activity and gene expression of GCL, a key rate-limiting enzyme in GSH synthesis (Zheng and Chen, 2007). It was further demonstrated that *de novo* synthesis of GSH was a prerequisite for curcumin to inhibit HSC activation (Zheng and Chen, 2007). In this study, we hypothesized that curcumin might protect the liver against CCl₄-induced injury and fibrosis by attenuating oxidative stress. Results in this report indicated that oral administration of curcumin not

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only increased the level of total hepatic GSH, but also significantly improve the ratio of GSH/GSSG in the liver. Further experiments revealed that curcumin enhanced the activity of GCL by inducing expression of the genes. Curcumin attenuated oxidative stress demonstrated by the reduction in the levels of lipid hydroperoxide in the CCl₄-rat model. These observations are supported by other studies. Treatment with curcumin (100mg/kg) prevented the drop in the content of hepatic GSH, diminished lipid peroxidation and minimized hepatocarcinogenesis in rats (Sreepriya and Bali, 2006). It bears emphasis that our results do not exclude any other mechanisms involved in the antioxidant capacity of curcumin and in the curcumin elevation of the level of hepatic GSH. The level of cellular GSH is mainly determined by GSH synthesis (GSH supply) and GSH-consuming (GSH demand). This current report focused on the effect of curcumin on GSH synthesis. Additional experiments are ongoing to evaluate the role of curcumin in regulating gene expression and activity of enzymes involved in consuming GSH, including GSH S-transferase and GSH peroxidase. In addition, we could not exclude the roles of any mechanisms and enzymes in the removal of lipid peroxidation products, which requires additional studies

Activation of HSC is triggered by various cytokines and chemokines, including the potent mitogens PDGF and EGF (Bachem et al., 1989b; Pinzani et al., 1989), as well as the fibrogenic factor TGF- β (Gressner et al., 2002), released from Kupffer cells and activated HSC (Friedman, 1999). The process of HSC activation is associated with sequential up-regulation of corresponding receptors, including PDGF- β R (Pinzani et al., 1996), EGFR (Komuves et al., 2000; Wong et al., 1994), type I and II TGF- β receptors (Friedman et al., 1994). We previously demonstrated that curcumin significantly suppressed gene expression of PDGF- β R, EGFR, type I and II TGF- β receptors in HSC *in vitro* (Zheng and Chen, 2004; Zheng and Chen, 2006; Zhou et al., 2007). Results in this

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report further confirmed the *in vivo* effect of curcumin on the suppression of expression of these genes in the CCl₄-rat model demonstrated by real-time PCR analyses and immunohistochemical assays. In addition, HSC activation coincides with a dramatic reduction in the abundance of PPAR γ (Marra et al., 2000; Miyahara et al., 2000), which might play an important role in hepatic fibrogenesis. We previously demonstrated that curcumin dramatically induced gene expression of PPAR γ in activated HSC, which facilitated its trans-activation activity, leading to the inhibition of HSC proliferation, the induction of apoptosis and the suppression of ECM production (Xu et al., 2003; Zheng and Chen, 2004). It was further reported that the interruption of the signaling pathways for TGF- β , PDGF and EGF by curcumin was required for the curcumin induction of gene expression of PPAR γ *in vitro* (Zheng and Chen, 2006; Zhou et al., 2007). It was of interest to verify that the treatment with curcumin diminished the role of CCl₄ in the reduction of the number of PPAR γ -positive HSC in the rat model.

In summary, the results from this study supported our initial hypothesis and demonstrated that curcumin protected the rat liver from CCl₄-caused injury and fibrogenesis *in vivo* by suppressing hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSC activation. These results confirm and extend our prior *in vitro* observations and provide novel insights into the mechanisms of curcumin in the protection of the liver. Our results suggest that curcumin might be a therapeutic anti-fibrogenic candidate for the treatment of hepatic fibrosis.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 Curcumin protects the liver from CCl₄-caused injury and fibrogenesis in the CCl₄-rat model. Rats were grouped, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). A piece of the liver tissue from each rat in the CCl₄-rat model was fixed with formalin and embedded in paraffin. Thin sections were cut and stained with hematoxylin-eosin (H & E) (**A**), Sirius red (**B**), and immuno-fluorescence using antibodies against α -SMA (**C**). Representative views from each group (n=6/group) were presented (original 25X). The numbers in the views represent the groups of rats in the experiments. Arrows indicated collagen deposition stained by Sirius red (**B**), or area positively labeled with α -SMA (**C**).

Figure 2 Curcumin suppresses inflammation in the CCl₄-rat model. Rats were grouped, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). A piece of liver tissue or blood was collected from each rat in the rat model. Levels of IFN- γ (**A**), TNF- α (**B**), and IL-6 (**C**) in the liver and in serum were determined by ELISA as described in "Materials and Methods". Values were expressed as mean \pm S.D. (n=6/group). *P<0.05 versus the level in Group #1 or #2 (the 1st or 2nd column on the left side); ‡P<0.05 versus the level in Group 3 (the 3rd column).

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Figure 3 Curcumin reduces the levels of PDGF and TGF- β in the CCl₄-rat model. Rats were grouped, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). Levels of PDGF-BB and TGF- β in the liver (**A**) or in serum (**B**) were determined by ELISA as described in "Materials and Methods". Values were expressed as mean \pm S.D. (n=6/group). *P<0.05 versus the level in Group #1 or 2 (the 1st or 2nd column on the left side); † P<0.05 versus the level in Group 3 (the 3rd column).

Figure 4 Curcumin modulates the expression of genes related to HSC growth and fibrogenesis. Total RNA was prepared from liver tissues from each group of rats, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). The steady state levels of mRNA in the liver were analyzed by real-time PCR assays. (**A**). The mRNA levels of genes relevant to cell growth, including PDGF- β R, EGFR and PPAR γ ; (**B**). The mRNA levels of genes relevant to fibrogenesis, including α -SMA, α (I) collagen, fibronectin (FN), T β -RII and T β -RI. Values were presented as means \pm S.D. (n=6). GAPDH was used as an invariant internal control for calculating mRNA fold changes. *p<0.05 versus the level in Group #1 or #2 (the 1st or 2nd columns on the left side). † P<0.05 versus the level in Group 3 (the 3rd column).

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Figure 5 Curcumin reduces the number of activated HSC doubly labeled with antibodies against PDGF- β R, or EGFR, and α -SMA in the CCl₄-rat model. Rats were grouped, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). A piece of the liver tissue from each rat in the CCl₄-rat model was fixed with formalin and embedded in paraffin. Thin sections were doubly stained with primary antibodies against PDGF- β R (**A**), or EGFR (**B**), plus antibodies against α -SMA and subsequently with goat anti-rabbit rhodamine conjugated secondary antibodies and donkey anti-mouse Alexor Fluor 488 conjugated secondary antibodies. Sections were viewed under a Bio-Rad MRC 1024 laser confocal microscope to visualize PDGF- β R-positive cells (the upper views with red fluorescence in **Panel A**), EGFR-positive cells (the upper views with red fluorescence in **Panel B**), and α -SMA-positive cells (the middle views with green fluorescence in **Panel A & B**). The merged images were presented in the lower views in **both panels**. Representative fluorescent images from each group (n=6) of the rat model were presented. Arrows indicated examples of positively stained cells (original 25X).

Figure 6 Curcumin reduces the number of activated HSC doubly labeled with antibodies against T β -RI, or T β -RII, and α -SMA in the CCl₄-rat model. Rats were grouped, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). A piece of the liver from each group in the CCl₄-rat model was fixed with formalin and embedded in paraffin. Thin sections were doubly stained with primary antibodies against T β -RI (**A**), or T β -RII (**B**), plus antibodies against α -SMA and

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subsequently with goat anti-rabbit rhodamine conjugated secondary antibodies and donkey anti-mouse Alexor Fluor 488 conjugated secondary antibodies. Sections were viewed under a Bio-Rad MRC 1024 laser confocal microscope to visualize T β -RI-positive cells (the upper views with red fluorescence in **Panel A**), T β -RII-positive cells (the upper views with red fluorescence in **Panel B**), and α -SMA-positive cells (the middle views with green fluorescence in **Panel A & B**). The merged images were presented in the lower views in **both panels**. Representative fluorescent images from each group (n=6) of the rat model were presented. Arrows indicated examples of positively stained cells (original 25X).

Figure 7 Curcumin eliminates the role of CCl₄ in the reduction of the number of PPAR γ -positive cells in the CCl₄-rat model. Rats were grouped, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). A piece of the liver from each rat in the CCl₄-rat model was fixed with formalin and embedded in paraffin. Thin sections were doubly stained with primary antibodies against PPAR γ and antibodies against α -SMA and subsequently with goat anti-rabbit rhodamine conjugated secondary antibodies and donkey anti-mouse Alexor Fluor 488 conjugated secondary antibodies. Sections were viewed under a Bio-Rad MRC 1024 laser confocal microscope to visualize PPAR γ -positive cells (the upper views with red fluorescence) and α -SMA-positive cells (the middle views with green fluorescence) in the liver tissues. The merged images were presented in the lower views. Representative fluorescent images from each group (n=6) of the rat model were presented. Arrows indicated examples of positively stained cells (original 60X).

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Figure 8 Curcumin increases the levels of hepatic GSH and reduces the production of hepatic lipid hydroperoxide in the CCl₄-rat model. A piece of the liver tissue was collected from each rat in all of the groups, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). Levels of hepatic GSH (**A**), the ratio of GSH/GSSG (**B**) and lipid hydroperoxide (**C**) were determined as described in "Materials and Methods". Values were expressed as mean \pm S.D. (n=6). *P<0.05 versus the level in Group #1 (the 1st column on the left side); **P<0.05 versus the level in Group #3 (the 3rd column).

Figure 9 Curcumin eliminates the inhibitory effect of CCl₄ on the GCL activity by inducing gene expression of GCL in the rat model

A piece of the liver tissue was collected from each rat in all of the groups, i.e. Group #1, the vehicle control (no CCl₄, no curcumin); Group #2, the curcumin control (no CCl₄); Group #3, the CCl₄ control (with CCl₄, no curcumin); Group #4, the curcumin-treated group (200 mg/kg + CCl₄); Group #5, the curcumin-treated group (400 mg/kg + CCl₄). (**A**). Determination of hepatic GCL activity. Values were presented as means \pm S.D. (n=6). *P<0.05 versus the activity in Group #1. †P<0.05 versus the activity in Group #3. (**B**). Real-time PCR analyses of the mRNA levels of GCLc and GCLm. Values were presented as means \pm S.D. (n=6). GAPDH was used as an invariant internal control for calculating mRNA fold changes. *P<0.05 versus the level in Group #1; †P<0.05 versus the level in Group #3; (**C**). Western blotting analyses of the protein abundance of GCLc and GCLm. β -actin was used as an invariant control for equal loading. Representatives of three independent experiments were shown.

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Table 1 Determination of the content of hepatic hydroxylproline in the rat model

	Group #1	Group #2	Group #3	Group #4	Group #5
Hydroxyproline ($\mu\text{mol/g}$)	3.51 \pm 0.80	3.11 \pm 0.41	5.91 \pm 1.21 [*]	4.12 \pm 0.64 [‡]	3.98 \pm 0.93 [‡]

*p>0.05, vs. Group #1. ‡p>0.05, vs. Group #3. Values were expressed as mean \pm S.D. (n=6).

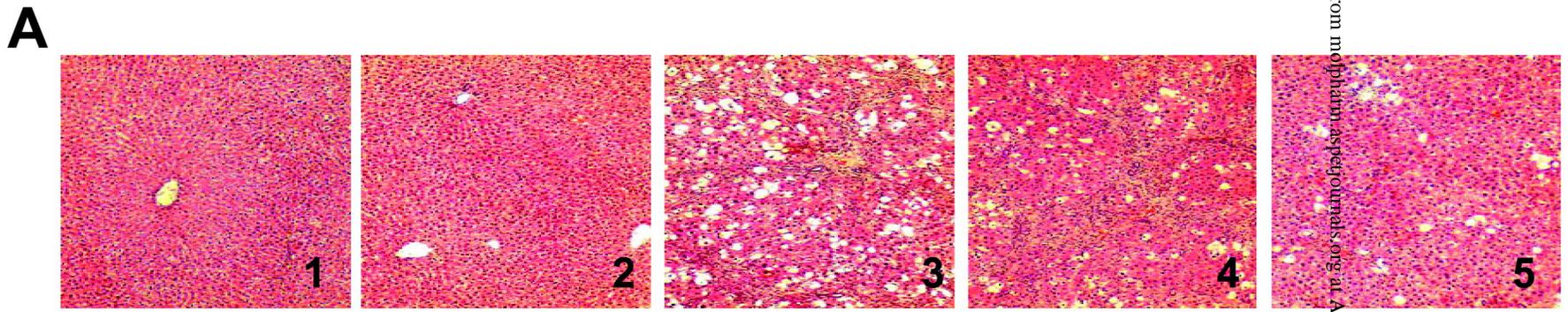
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Table 2 Quantitation of ALP, ALT and AST in the sera from rats in the model with CCl₄-caused hepatic injury

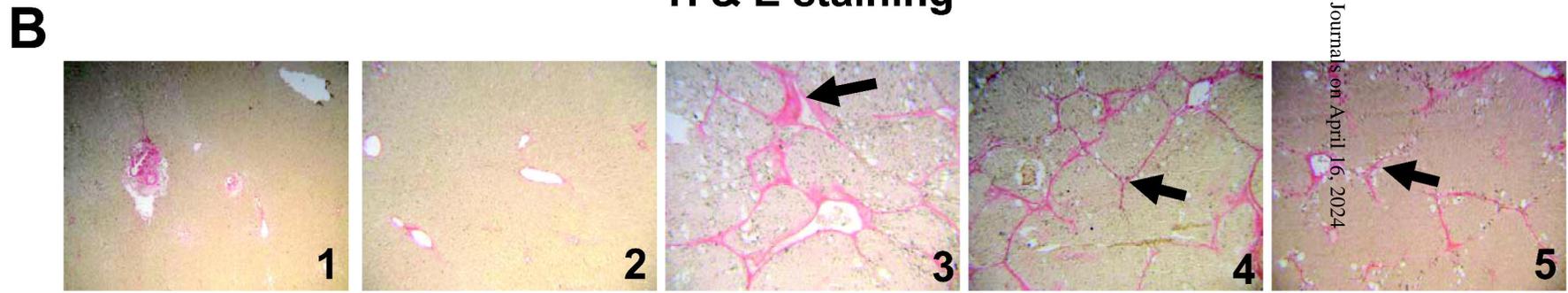
Groups	ALP(U/L)	ALT(U/L)	AST(U/L)
#1 Vehicle control	108.33 ± 37.59	45 ± 7.54	137.67 ± 50.9
#2 curcumin control	82.83 ± 41.85	35.67 ± 11.04	96.5 ± 30.41
#3 CCl ₄ control	370.17 ± 11.71*	802.17 ± 13.21*	1771.13 ± 96.08*
#4 Curcumin 200 mg/kg	263.5 ± 15.11**	511.83 ± 18.63**	1046 ± 20.53**
#5 Curcumin 400 mg/kg	249.33 ± 14.85**	482.17 ± 48.42**	1097.83 ± 94.62**

*p<0.05, vs. the vehicle Group #1. **p<0.05, vs. the rat Group #3 injected with CCl₄.

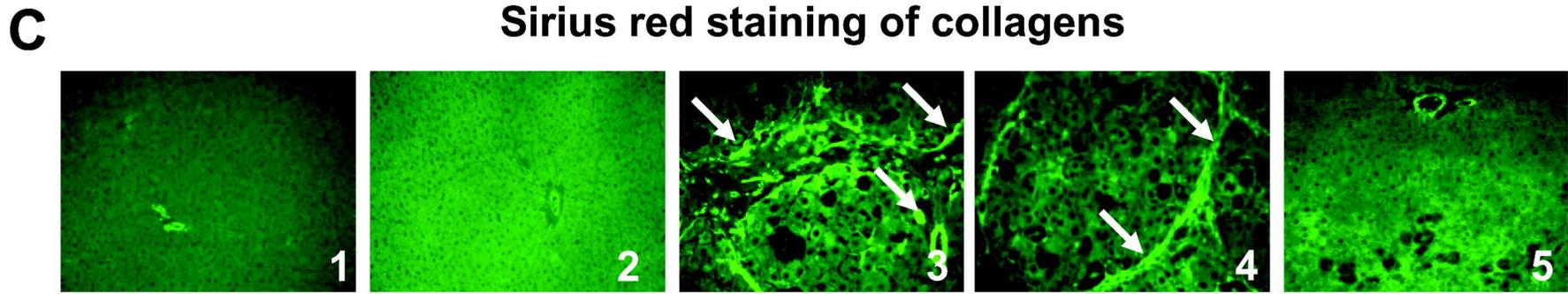
Blood was collected from rats in each group in the rat model through heart puncture when sacrificed. After coagulation, the activities of serum AST, ALT and ALP were analyzed. *P<0.05 versus the level in Group #1 or #2; **P<0.05 versus the level in Group #3. Values were expressed as mean ± S.D. (n=6).



H & E staining



Sirius red staining of collagens



Immuno-staining of α -SMA-positive cells in rat livers

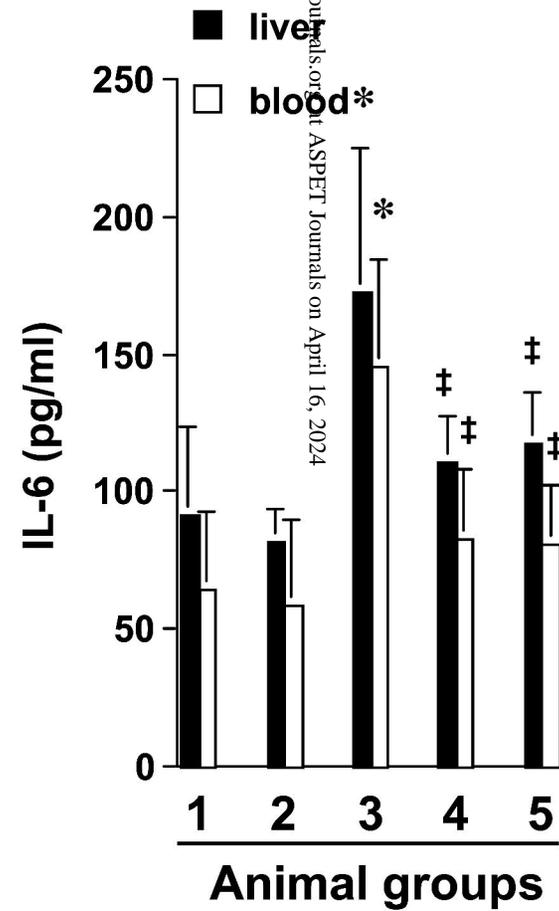
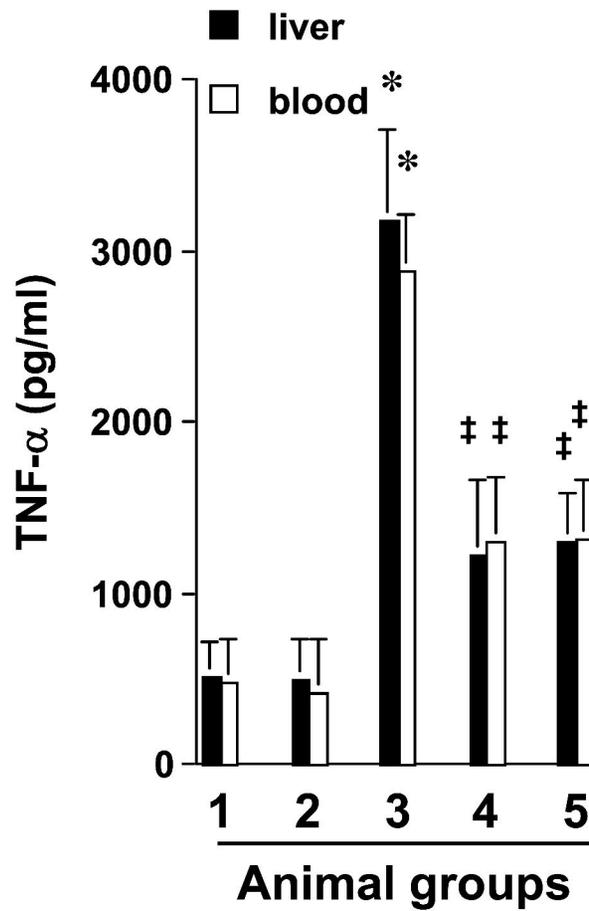
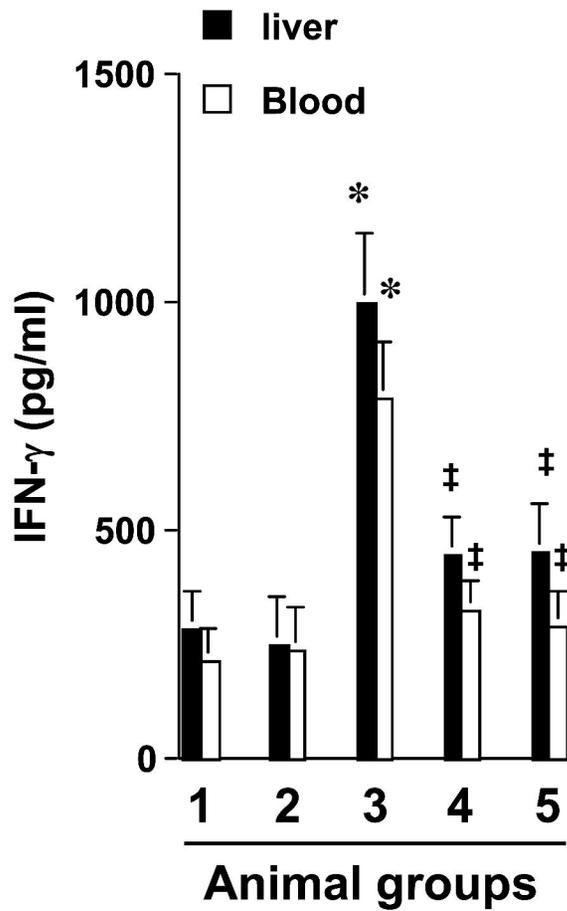


Fig. 2

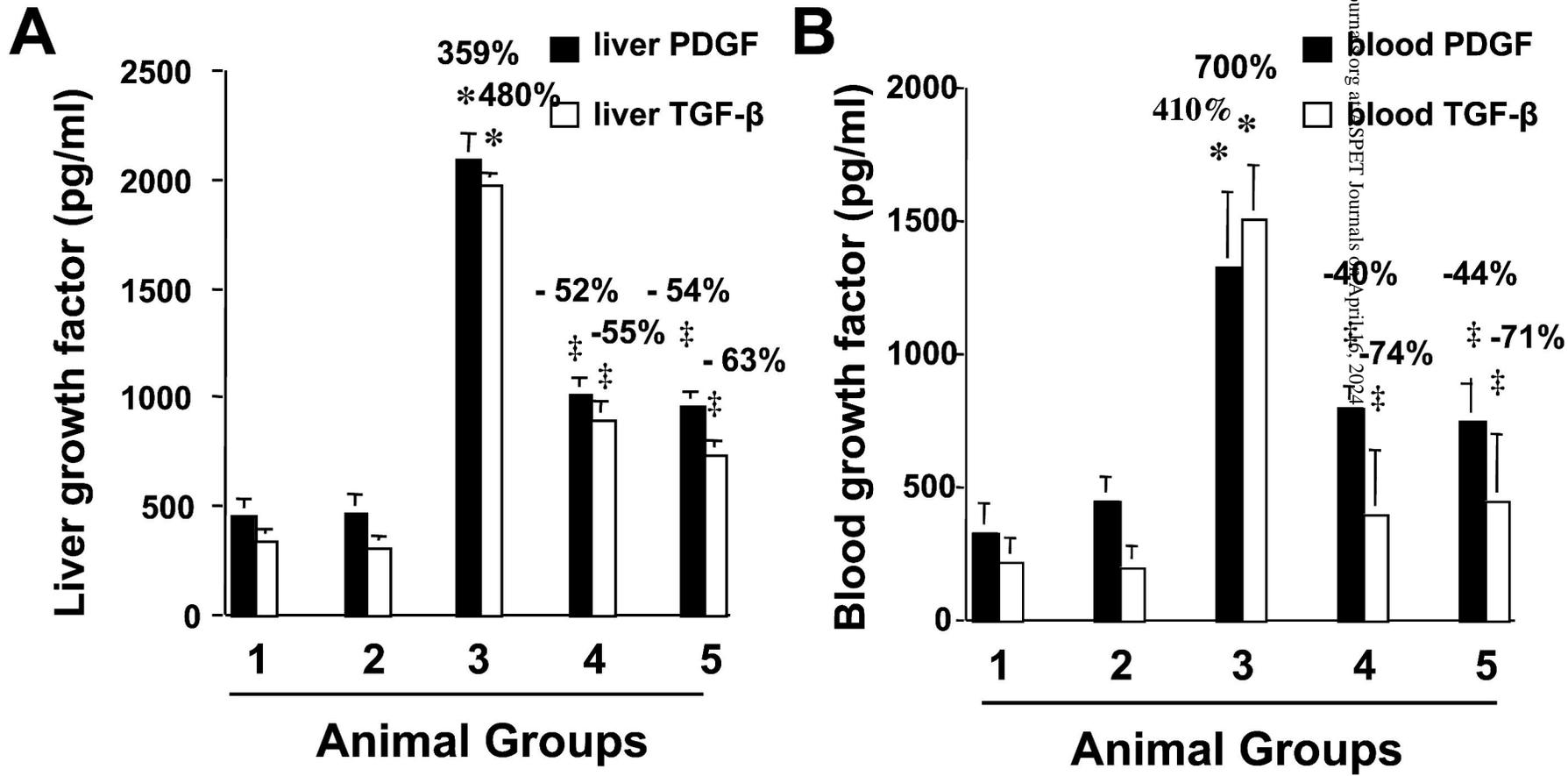


Fig. 3

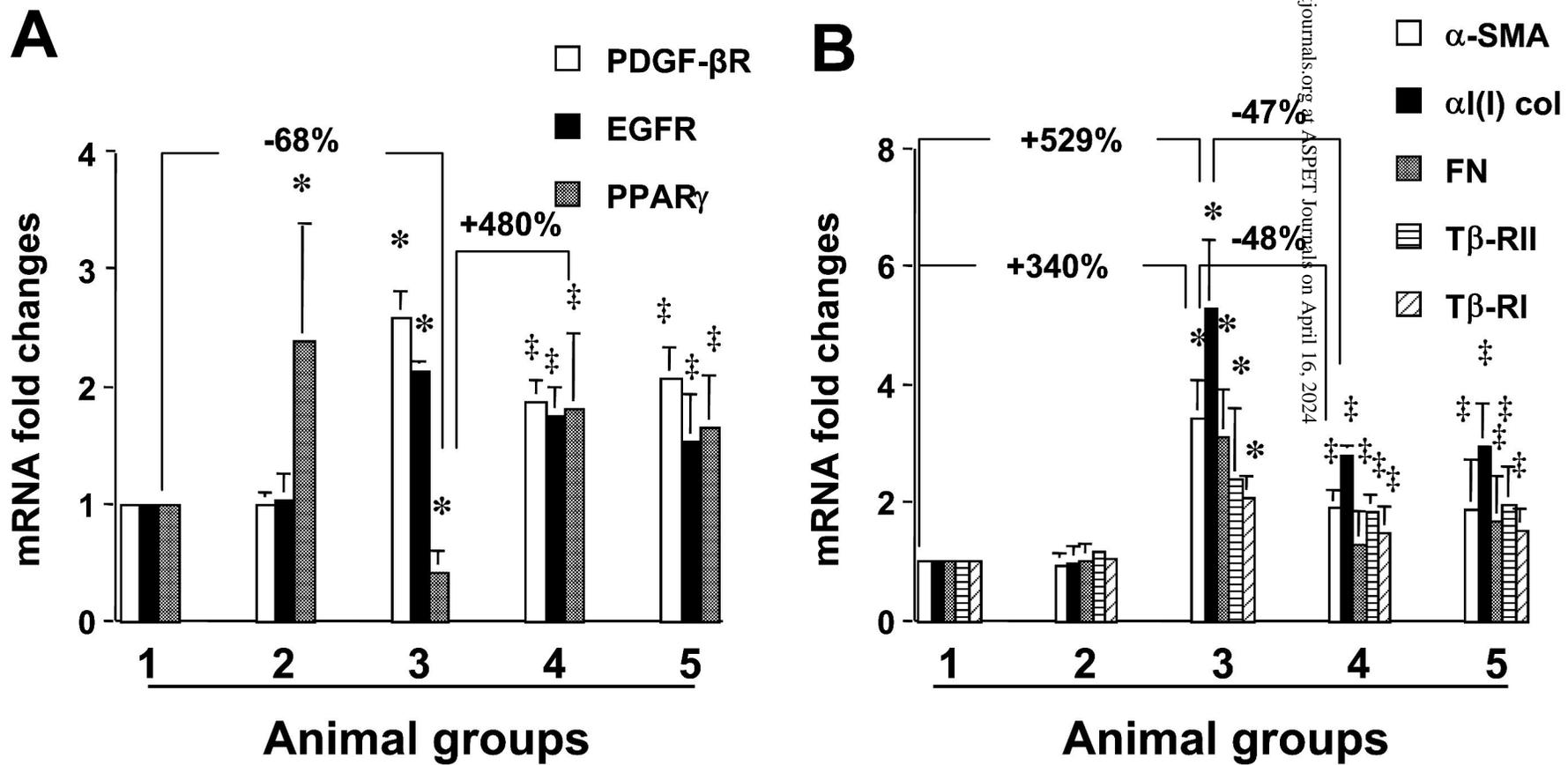


Fig. 4

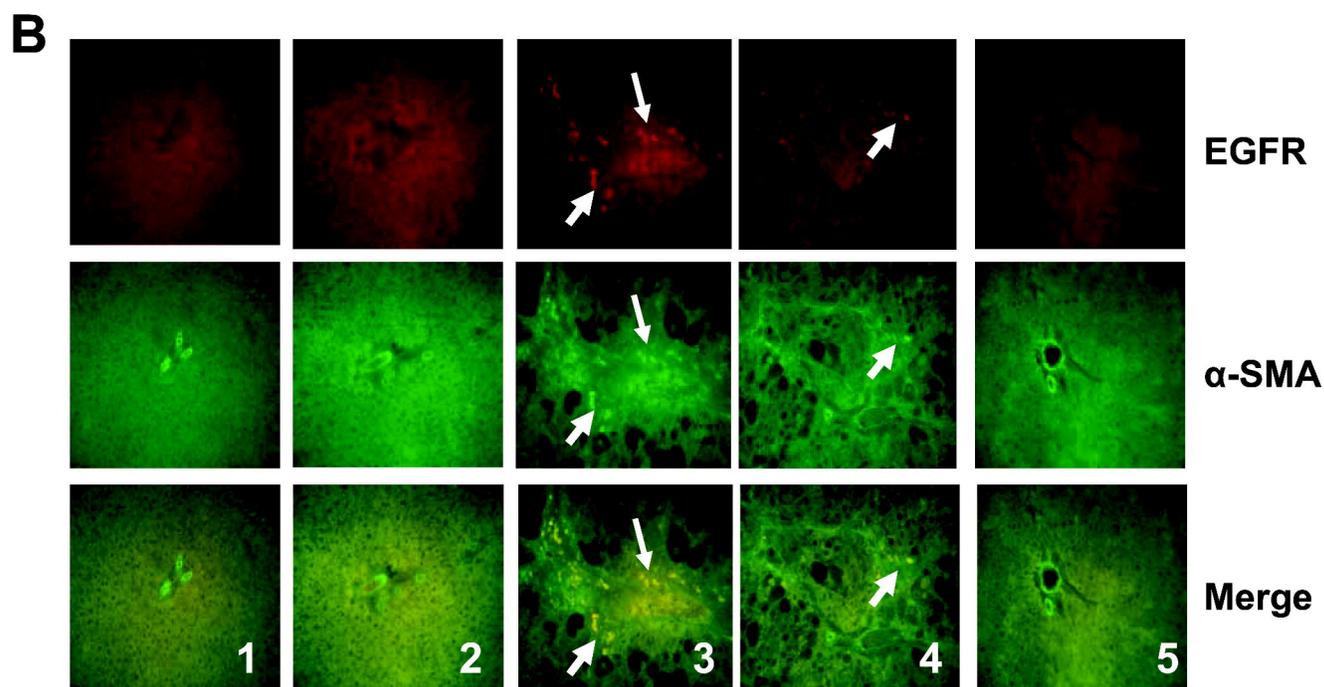
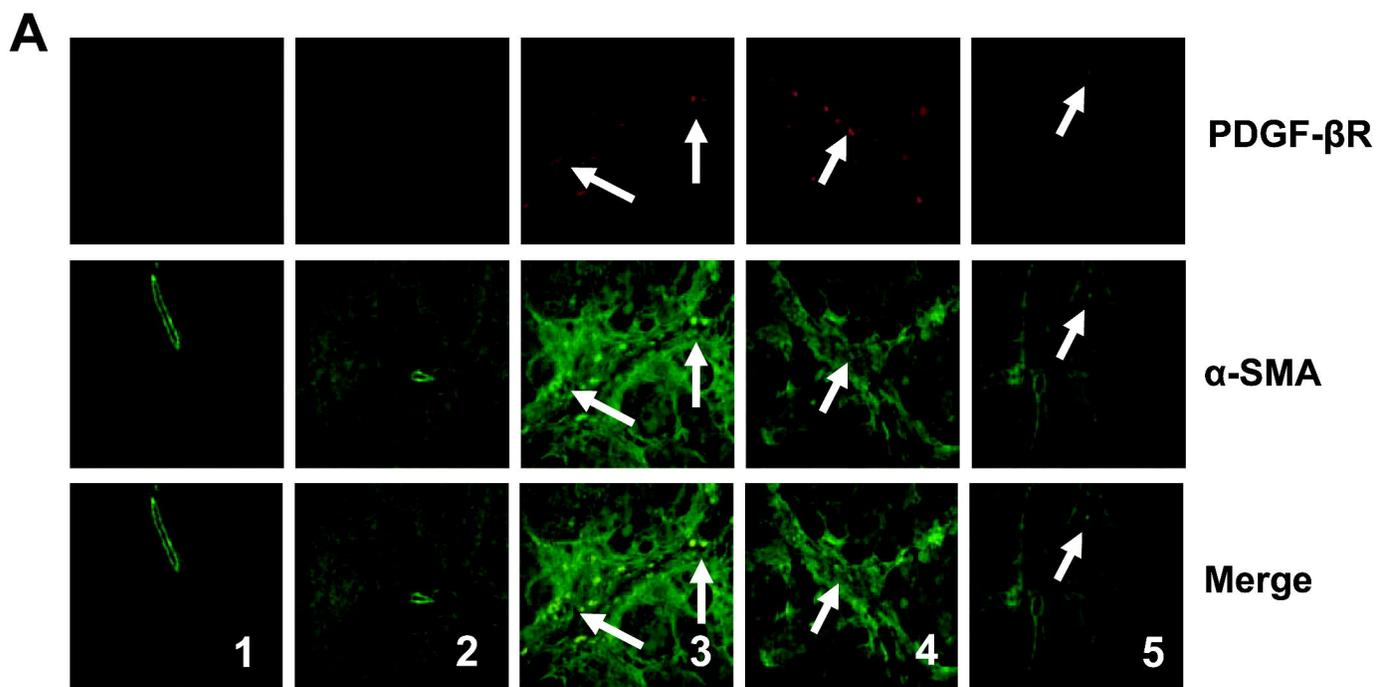
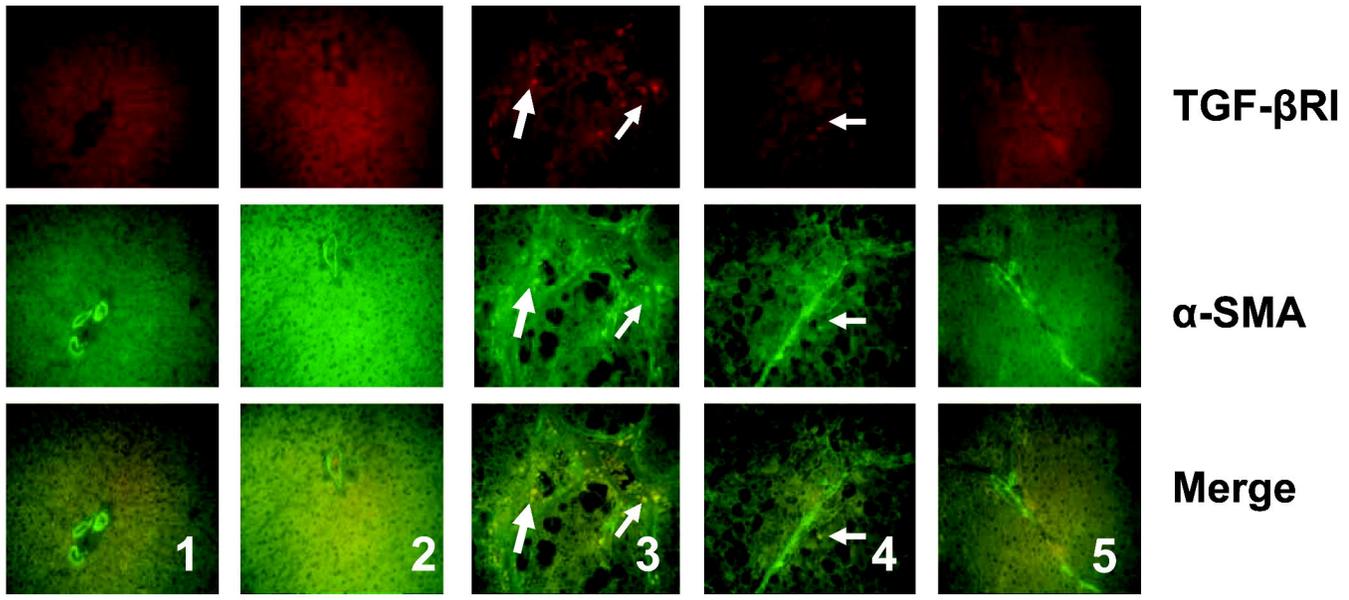
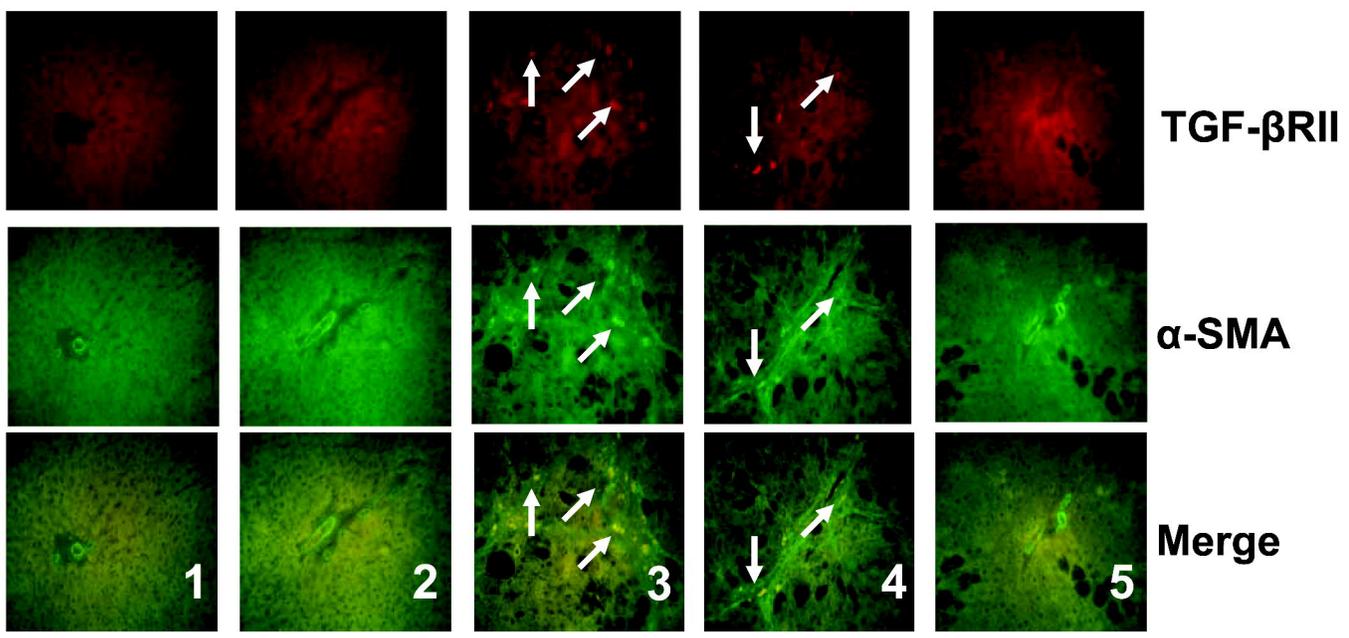


Fig. 5

A**B****Fig. 6**

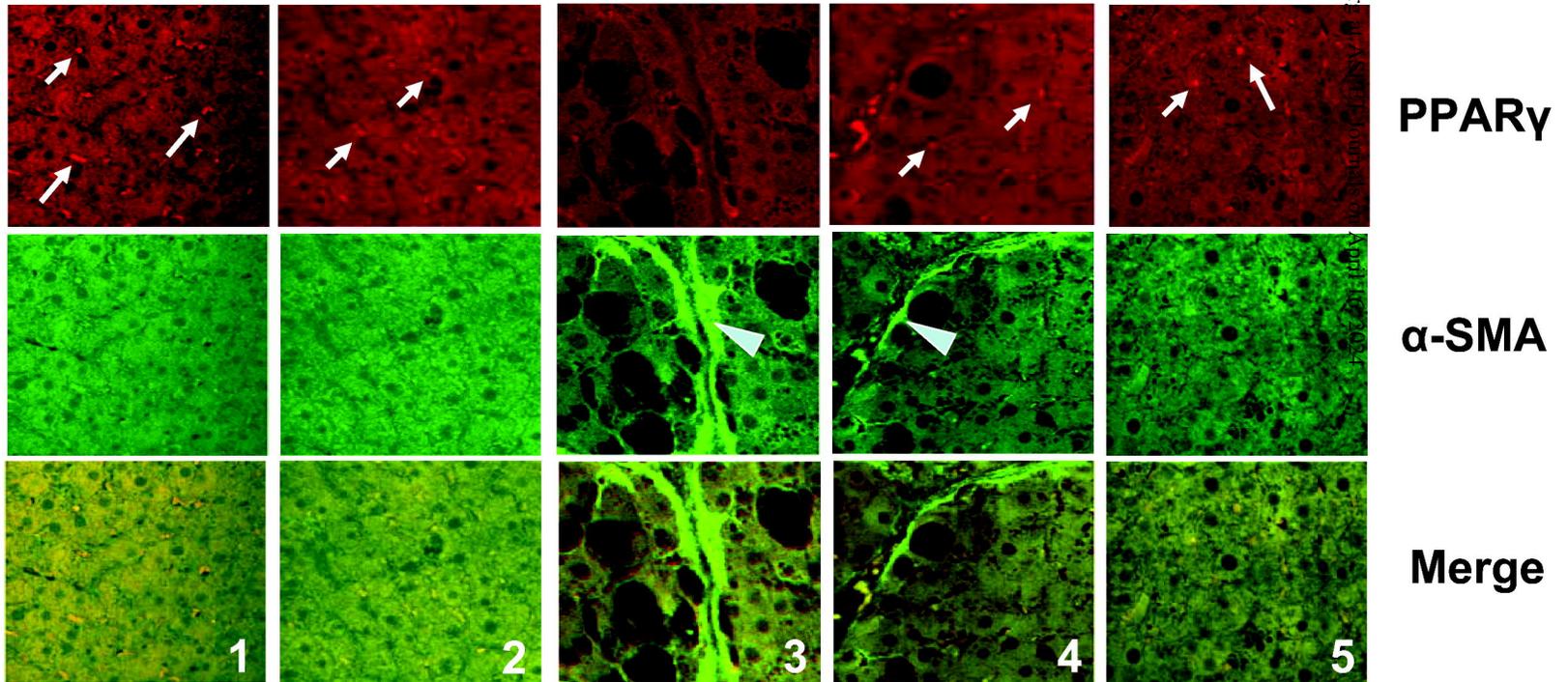


Fig. 7

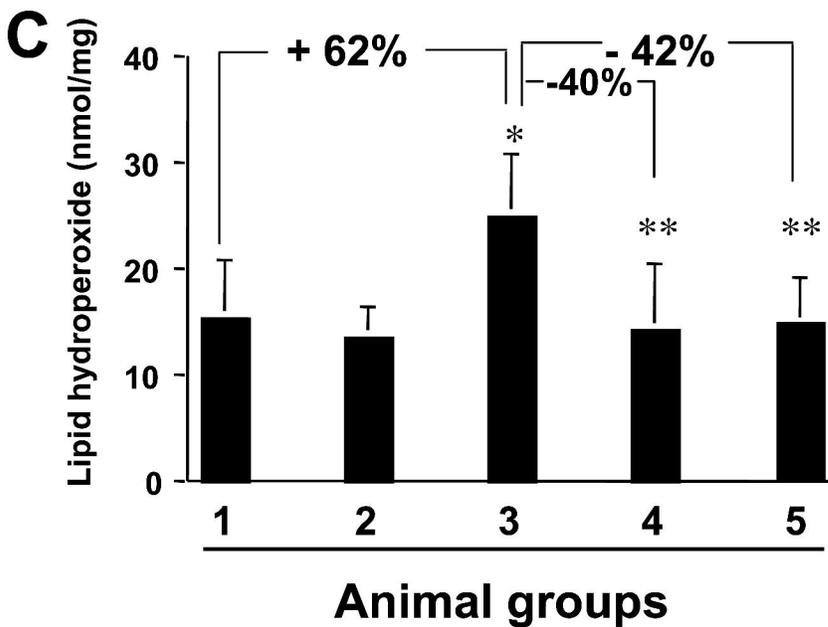
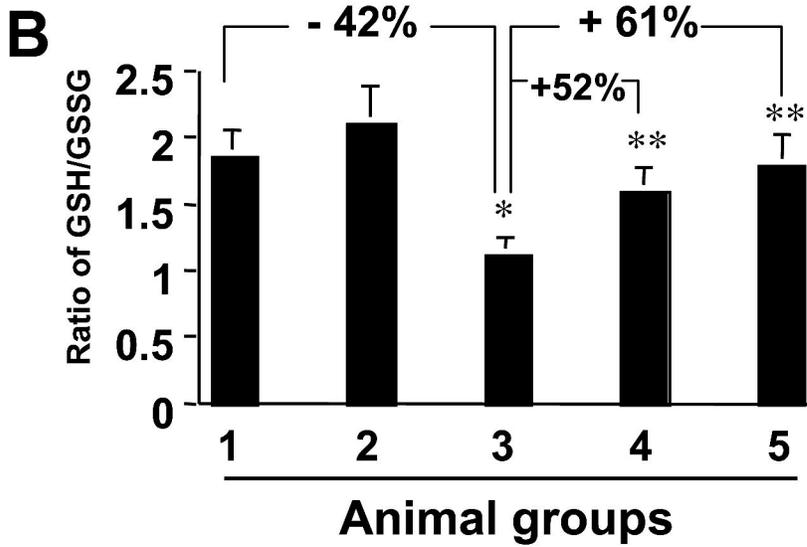
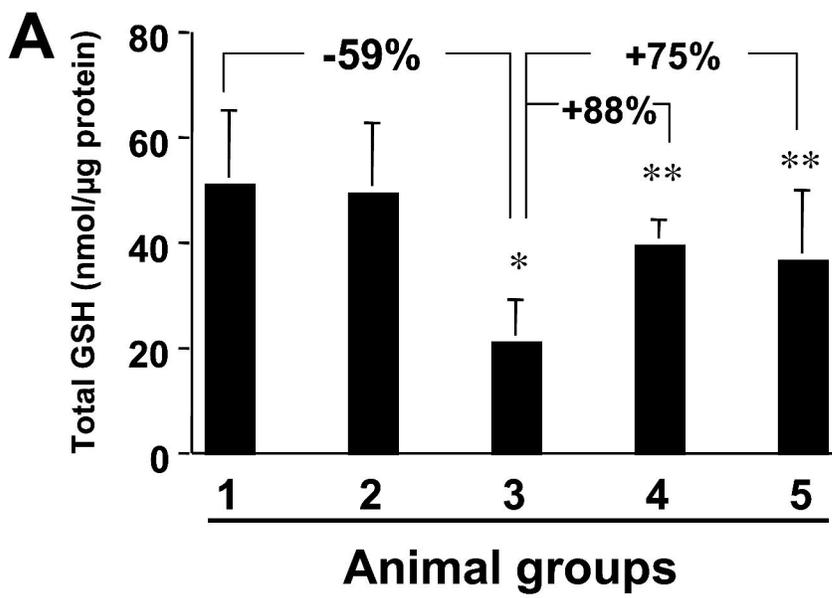


Fig. 8

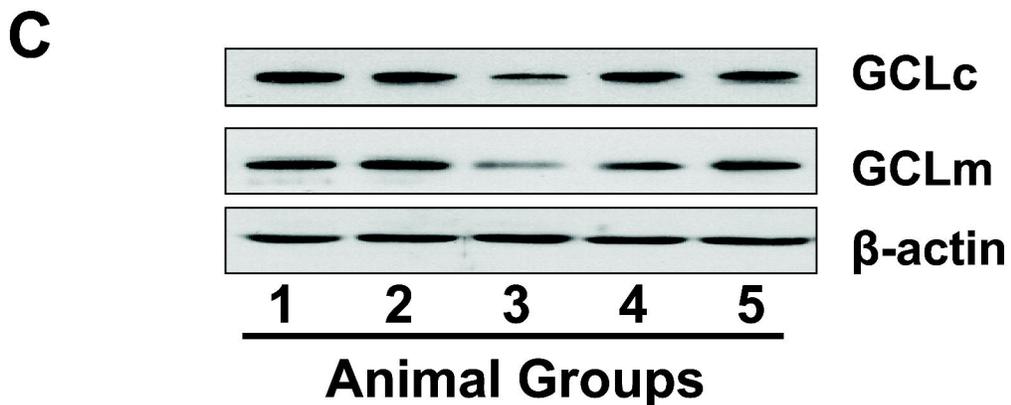
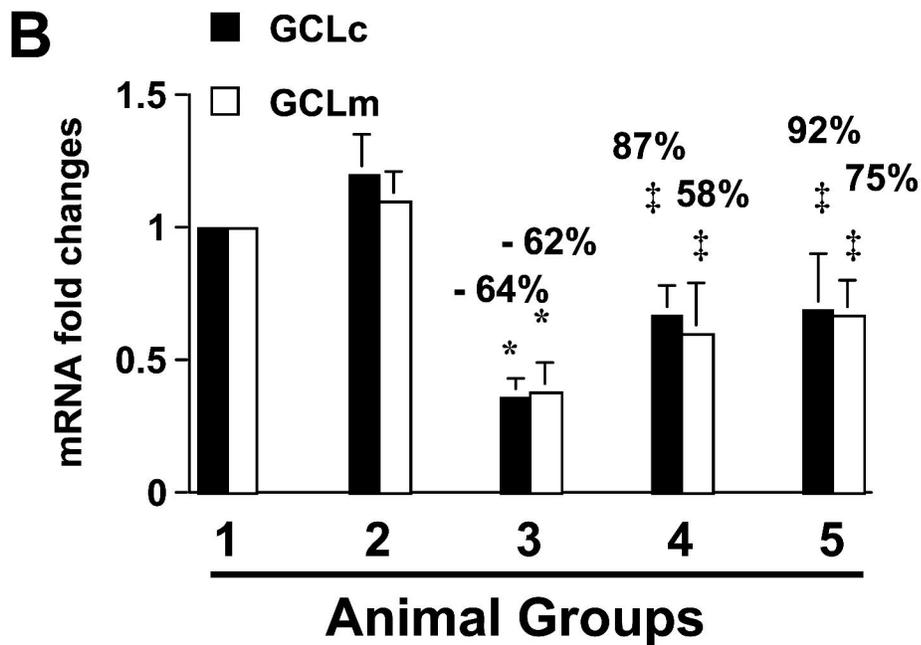
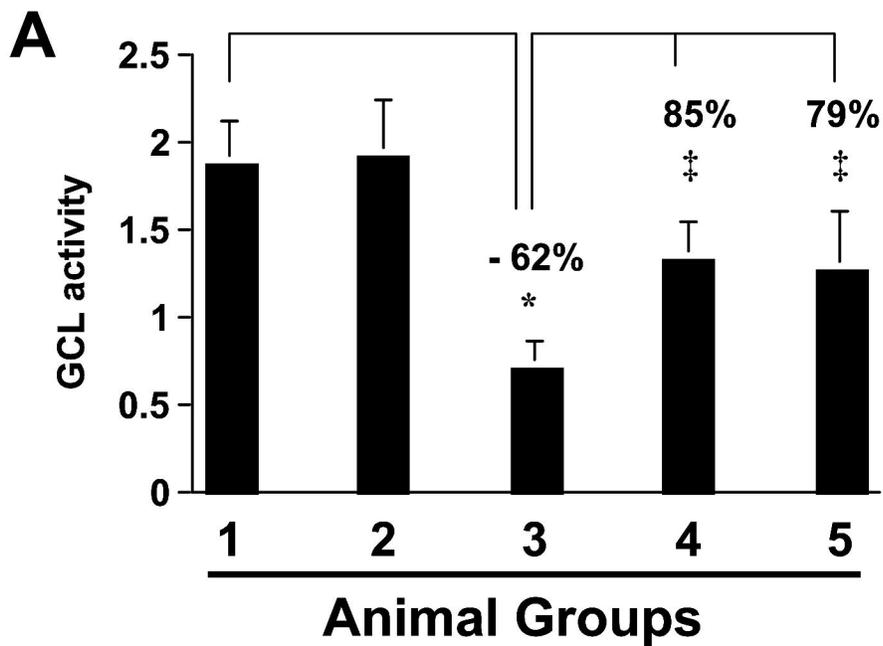


Fig. 9