Histol Histopathol (2010) 25: 819-830

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

The role of prostaglandin E_2 in acute acetaminophen hepatotoxicity in mice

Ivan Ćavar[1], Tomislav Kelava[2], Katarina Vukojević[1,3], Mirna Saraga-Babić[3] and Filip Čulo[1,2]

[1]Department of Physiology, School of Medicine, University of Mostar, Bosnia and Herzegovina, [2]Department of Physiology, School of Medicine, University of Zagreb, Croatia and [3]Department of Anatomy, Histology and Embryology, School of Medicine, University of Split, Croatia

Summary. Prostaglandin E_2 (PGE_2), which is synthesized by many cell types, has a cytoprotective effect in the gastrointestinal tract and in several other tissues and cells. On the other hand, overdose or chronic use of a high dose of acetaminophen (Paracetamol, APAP) is a major cause of acute liver failure in the western world. These observations prompted us to investigate whether PGE_2 plays a role in host defence to toxic effect of APAP. (ČBAT6T6xC57Bl/6)F_1 hybrid mice of both sexes were intoxicated with a single lethal or high sublethal dose of APAP, which was administered to animals by oral gavage. Stabile analogue of PGE_2, 16,16-dimethyl PGE_2 (dmPGE_2), or inhibitor of its production, CAY10526, were given intraperitoneally (i.p.) 30 minutes before or 2 hours after APAP administration. The toxicity of APAP was determined by observing the survival of mice during 48 hours, by measuring concentration of alanine-aminotransferase (ALT) in plasma 20-22 hours after APAP administration and by liver histology. The results have shown that PGE_2 exhibits a strong hepatoprotective effect when it is given to mice either before or after APAP, while CAY10526 demonstrated mainly the opposite effect. Immunohistochemical or immunofluorescent examinations in the liver tissue generally support these findings, suggesting that PGE_2 inhibited APAP-induced activation of nuclear factor kappa B (NF-κB). Similarly, PGE_2 down regulated the activity of inducible nitric oxide synthase (iNOS), which was up regulated by APAP. Thus, by these and perhaps by other mechanisms, PGE_2 contributes to the defence of the organism to noxious effects of xenobiotics on the liver.

Key words: Prostaglandin E_2, Acetaminophen, Liver injury, NF-κB, Immunohistochemistry

Offprint requests to: Ivan Ćavar, Department of Physiology, School of Medicine, University of Mostar, Bijeli brijeb b.b., 88000 Mostar, Bosnia and Herzegovina. e-mail: ivancavarsb@yahoo.com

Introduction

Acetaminophen (Paracetamol, N-acetyl-p-aminophenol, APAP), the most commonly used analgesic and antipyretic drug, is very safe at therapeutic doses. However, overdose or chronic use of a high dose of APAP has been shown as a major cause of acute liver failure in the western world (reviewed in Bernal 2003; Lee, 2003). After an overdose of APAP, the reactive metabolite, *N-acetyl-p*-benzoquinone imine (NAPQI), is generated by hepatic cytochromes P450 (CYPs). Although, the precise biochemical mechanism of cell death is not fully understood it is generally recognized that NAPQI triggers hepatic toxicity via mitochondria injury, apoptosis and cell necrosis. These effects of NAPQI are mediated through its covalently binding with nucleophilic macromolecules such as DNA or proteins, glutathione (GSH) depletion, lipid peroxidation and generation of oxidative stress molecules (Jaeschke, 2000; James et al., 2003; Mazer and Perrone, 2008). It is believed that inflammatory mechanisms play a role in the later phases of liver APAP toxicity (O'Connor and Bennet, 2005). Thus, it was shown that nuclear factor kappa B (NF-κB) is also activated during APAP-induced hepatotoxicity (Bauer et al., 2000; Dambach et al., 2006). Oxidative stress has been shown to activate signal transduction pathways involving transcription factors such as NF-κB and activator protein-1 (AP-1) (Jaeschke, 2000; Janssen-Heininger et al., 2000). NF-κB is known to regulate the expression of genes controlling inflammatory mediators, including inducible nitric oxide synthase (iNOS) and consequent production of nitric oxide (NO), which is a highly reactive oxidant produced in the liver in response to different inflammatory stimuli and has been implicated in hepatotoxicity (Gardner et al., 2002; James et al., 2003; Kamanaka et al., 2003).

Prostaglandins (PGs) are lipid-derived autacoids generated by sequential metabolism of arachidonic acid by the cyclooxygenase (COX) and prostaglandin

synthase enzymes. PGs are ubiquitously produced and have been implicated in a broad array of diseases, including cancer, inflammation, cardiovascular disease and hypertension (reviewed in Hata and Breyer, 2004; reviewed in Matsuoka and Narumiya, 2007). Prostaglandin E_2 (PGE_2) is produced by many cells of the body and exerts its actions by binding to one (or a combination) of its four subtypes of receptor, EP1, EP2, EP3 and EP4 (Harris et al., 2002). PGE_2 is considered to be very important for normal physiological functions, especially for the function of gastrointestinal tract (Dey et al., 2006) and kidney (Hao and Breyer, 2008). Thus, in the stomach of rat PGE_2 was essential for conferring protection against indomethacin or ethanol-induced injury (Araki et al., 2000; Suzuki et al., 2001). Protective effects of exogenous PGE_2 and prostacyclin (PGI_2) has been demonstrated in various models of liver injury induced with lipopolysaccharide (LPS), D-galactosamine (DGalN), concanavalin A (Con A), carbon tetrachloride (CCl_4), virus and ischemia-reperfusion (reviewed in Quiroga and Prieto, 1993; Yin et al., 2007). The protective effect of PGE_2 was also observed, but not directly proven, in APAP-induced liver injury in mice (Renić et al., 1995; Reilly et al., 2001). Various mechanisms for cytoprotective effects of PGE_2 are proposed (reviewed in Quiroga and Prieto, 1993). Data *in vitro* have revealed plenty of cell signaling pathways by which this protection could be mediated. Most often investigated is the effect of PGE_2 on activation of NF-κB by which it may have an influence on the synthesis of inflammatory hepatotoxic cytokines and generation of oxidative radicals (Tran-Thi et al., 1995; Laskin et al., 2001; Dambach et al., 2006; Ogawa et al., 2009), activation of iNOS (Laskin et al., 2001; James et al., 2003), stimulation of synthesis of inhibitory (suppressive) cytokine – IL-10 (Cheon et al., 2006), endogenous antioxidants (Enomoto et al., 2001) and cyclic adenosine monophosphate (cAMP), which has various immunosuppressive and anti-inflammatory effects (Bourne et al., 1974; Nakano et al., 1994). However, some of these mechanisms are far from being definitively proven, since their role in toxicity of APAP is not firmly established and the obtained effects of PGE_2 were sometimes contradictory. This particularly relates to effects on activation of NF-κB and iNOS.

Based on these data, the present studies aimed to investigate the role of exogenously applied PGE_2 and its derivatives on APAP-induced hepatotoxicity *in vivo*, as well as to examine their effects on expression of NF-κB and iNOS in liver tissue.

Materials and methods

Animal model

Male or female (CBAT6T6xC57Bl/6)F_1 hybrid mice aged 12-16 weeks and weighing 20-30 g were used in all experiments. They were raised in an animal facility unit at the Department of Physiology, School of Medicine, University of Zagreb. The animal colony unit had regulated 12 h light/dark cycle and the temperature and relative humidity in the animal room were 22±2°C and 50±5%, respectively. The cages were sanitized twice weekly and mice were allowed free access to tap water and standard mouse chow diet (No. 4RF21, Diet Standard, Milano, Italy). All animal protocols were approved by the Ethics Committee of the University of Zagreb, School of Medicine (Zagreb, Croatia).

Chemicals and treatments of animals

Pure APAP substance was a kind gift from Belupo (Koprivnica, Croatia). Phenobarbitone-sodium was obtained from Kemika (Zagreb, Croatia). Since the PGE_2 is rapidly conversed to an inactive metabolite, 13,14-dihydro-15-keto PGE_2, its stable structural analog, 16,16-dimethyl PGE_2 ($dmPGE_2$), was used for *in vivo* experiments. Stock solution of $dmPGE_2$ in methyl acetate (No. 14750, Cayman Chemical, Ann Arbor, MI, USA) was first evaporated under a gentle stream of nitrogen and the remaining substance was dissolved (1.0 mg/mL) in phosphate buffered saline (PBS, pH=7.2). $DmPGE_2$ was administered to animals (0.2 mg/kg, i.p.) 30 min before or 2 h after APAP. CAY10526 (an inhibitor of PGE_2 production through the selective modulation of microsomal PGE synthase-1 expression, mPGES-1) was supplied as a crystalline solid (No. 10010088, Cayman Chemical). Since the CAY10526 is sparingly soluble in aqueous buffers, it was first dissolved in an organic solvent, dimethyl formamide (DMF, 25 mg/mL), diluted in PBS (pH=7.2) and finally injected (2.0 mg/kg, i.p.) into animals 30 min before or 2 h after APAP. The doses of the drugs for application *in vivo* were chosen from scarce data in the literature or according to the toxicity data in our preliminary experiments, in which the effects of the drugs on survival of mice and gross macroscopic changes of liver and other visceral organs were observed. Animals in control groups received appropriate vehicle. Survival of mice was followed for 48 h after APAP administration, since almost all mice either died within this period or fully recovered thereafter. For immunohistochemical or immunofluorescent examinations, PGE_2 or vehicle was given to mice 30 min before, and CAY10526 was given 2 h after APAP administration.

Assessment and measurement of hepatotoxicity induced with APAP

In order to induce hepatic cytochromes P450 (CYPs), mice were given phenobarbitone-sodium in drinking water for 7 days (0.3 g/L). Thereafter, mice were fasted overnight and APAP was given by oral gavage in a volume of 0.4 to 0.6 mL. APAP was dissolved under mild magnetic stirring in warm PBS, to which 1-2 drops of Tween 20 were added. Animals were allowed free access to food 4 h later (Guarner et al., 1988; Renić et al., 1995). To observe the survival of the mice, APAP was administered in a dose of 250-300 mg/kg, which in our previous experiments induced 43 to

72% mortality of untreated animals. To determine plasma alanine aminotransferase (ALT) and NO concentration in plasma, as well as for histopathological, immunohistochemical or immunofluorescent evaluation of liver slices and measurement of PGE_2 production by liver fragments, mice were treated with high sublethal dose of APAP (150 mg/kg). Experimental and control groups of mice contained 12-13 animals (for observation of survival) or 6-10 animals (for all other measurements).

Plasma ALT activity

ALT levels were determined 20-22 h after APAP administration. Mice were given 250 U heparin i.p. 15 min before bleeding and blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes. After centrifugation, separated plasma was stored at -80°C for 24 h before ALT determination. ALT concentrations were measured by standard laboratory techniques (Reniç et al., 1995).

Liver histology

Mice were sacrificed under light ether anesthesia by cervical dislocation 20-22 h after APAP administration. Liver lobes of each animal (9-10 animals per group) were fixed in 4% buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Thereafter, sections of tissue were cut at 5 mm on a rotary microtome, mounted on clean glass slides and dried overnight at 37°C. The sections were cleared, hydrated and stained with hematoxyllin and eosin. Microscopically, the liver damage was classified using arbitrary scale from 0 to 5 as follows: degree 0–there was no damage; degree 1–minimal lesions involving single to few necrotic cells; degree 2–mild lesions, 10-25% necrotic cells or mild diffuse degenerative changes; degree 3–moderate lesions, 25-40% necrotic or degenerative cells; degree 4–marked lesions, 40-50% necrotic or degenerative cells; degree 5–severe lesions, more than 50% necrotic or degenerative cells. Sections with scores higher than 2 were considered to exhibit significant liver injury (Silva et al., 2001).

Influence of CAY10526 on ex vivo production of PGE_2

Mice were sacrificed 6 h after APAP administration and samples of liver tissue, kept on ice, were minced in small fragments (1-2 mm^3) in PBS. After sedimentation at unit gravity, they were washed 2 times more in fresh PBS, transferred into preweighed tubes and centrifuged at 500 g at +4ºC for 3 min. The sediment was quickly weighed, resuspended in Minimal Essential Medium (MEM, 5 μl MEM/mg tissue) and incubated in a water bath at 37 ºC for 1 h. The samples were then centrifuged as above and supernatants stored at -80ºC until analysis. Concentration of PGE_2 was determined using appropriate PGE_2 EIA Kit according to the manufacturer's instructions (No. 514010, Cayman Chemical) (Culo et al., 1995).

Immunohistochemistry

Mice were sacrificed under light ether anesthesia by cervical dislocation 6 h after APAP administration. Immediately, liver lobes were taken from each animal (6 mice per group) and tissue samples were fixed in 4% paraformaldehyde in phosphate buffer and dehydrated in 100% ethanol. They were embedded in paraffin wax, serially sectioned as 7 μm thick sections and mounted on glass slides. After removing paraffin with xylene, the sections were rehydrated in ethanol and water. In order to quench endogenous peroxidase activity, sections were incubated for 10 min in 0.3% H_2O_2, washed in PBS and then cooked in sodium citrate buffer for 17 min at 95°C. After being cooled to room temperature, sections were incubated with diluted (1:100) polyclonal rabbit anti-NF-κB (p65 subunit) or anti-iNOS antibodies (No. sc-109 and sc-651, respectively, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at room temperature in a humidified chamber. After being washed with PBS, sections were incubated with a biotinylated secondary antibody (rabbit UniTect ABC Kit, Oncogene, Boston, Mass., USA) for 30 min at room temperature. Afterwards, sections were washed in PBS and incubated with avidin biotinylated horseradish peroxidase complex (ABC) for 30 min, washed again with PBS and stained with diaminobenzidine (DAB). Finally, sections were rinsed in distilled water, counterstained with hematoxyllin and dehydrated in ethanol and xylol. Cells positive to NF-κB had brown-stained cytoplasm or nuclei (depending of NF-κB activation) and cells positive to iNOS had brown-stained cytoplasm. Positive internal controls for NF-κB and iNOS staining were smooth muscle cells in vascular walls. Negative controls were prepared by substituting Dako ChemMate antibody diluent for secondary antibody. Images were captured with digital camera (SPOT Insight, Diagnostic Instruments, USA) mounted on an Olympus BX51 microscope using the SPOT software.

Immunohistochemical quantification

Liver tissue specimens of four experimental groups of mice were analyzed. The number of cells was evaluated quantitatively by two independent investigators and classified as negative (not stained) cells, nuclear positive for NF-κB counting and cytoplasm positive for iNOS counting. Counts were made along the length of the liver only for DAB staining sections. DP-SOFT version 3.1 software was used to divide each liver section into squares of 100x100 μm at 40x magnifications. The cells below the left and upper border of squares were not taken into account, but only those at the right and lower border. To avoid counting the same cell twice, we used every other consecutive section. The examination was performed on an Olympus BX51 microscope equipped with a DP11 digital camera.

Images were analyzed using DP-SOFT version 3.1 software.

Immunofluorescence

After deparaffinization and rehydration, the sections were treated in a microwave oven at 95°C for 17 minutes in sodium citrate buffer (pH 6.0) for antigen retrieval. After being cooled to room temperature, sections were washed in PBS and incubated with goat serum (Normal Goat Serum, X0907 DAKO, Glostrup, Denmark) for 1 h to block non-specific antibody binding. Sections were then incubated with primary antibodies for 1 h at room temperature. Polyclonal rabbit anti- p65 antibody from Santa Cruz Biotechnology (No. sc-109), diluted 1:100, was used. After multiple washes in PBS, sections were incubated for 1 h with diluted (1:200) secondary antibody (Texas Red, No. sc-2780, Santa Cruz Biotechnology). Following secondary antibody incubation, the sections were washed in PBS and counterstained with diamidino-2-phenylindole (DAPI) to stain nuclei. After final rinsing in PBS, sections were mounted, air-dried and coverslipped (Immuno-Mount, Shandon, Pittsburgh, PA, USA). Control of specificity included omitting primary antibody from the staining procedure. Sections were examined by using a fluorescence microscope (Olympus BX61, Tokyo, Japan). Immunofluorescent images were obtained with a digital camera (DP71) mounted on an Olympus BX61 microscope using the Olympus Cell software and Adobe Photoshop.

Measurement of NO in plasma

Mice were sacrificed under light ether anesthesia and plasma samples were taken 6 h after treatment of mice with APAP. Thereafter, samples were stored at -80°C until determination of NO synthesis. For quantification of total NO production, nitrate (NO_3^-) was first reduced into nitrite (NO_2^-) using copper-coated cadmium granules (NITRALYZER-II, Nitrate to Nitrite reduction kit, World Precision Instruments, Sarasota, FL, USA). Concentration of nitrite was measured by Griess reaction according to the manufacturer's instructions. Finally, absorbance readings were performed with the use of an automated microplate reader (Dynatech MR 5000, Dynatech Technology, Inc., Horsham, PA, USA) at 540 nm.

Statistical analysis

Results are expressed as mean ± SEM. Differences in survival between groups of mice were compared by chi-square test using Yate's correction when indicated. Statistical comparisons between two groups were made using a Student's t-test. Comparisons between multiple groups were carried out using one-way analysis of variance (ANOVA) with a *post hoc* test of significance between individual groups. Significance was accepted at $p<0.05$.

Results

Effects of $dmPGE_2$ and CAY10526 on APAP-induced mortality and plasma ALT concentration in mice

To determine the survival of animals, mice were treated with 300 mg/kg of APAP. $DmPGE_2$ (0.2 mg/kg, i.p.) and CAY10526 (2.0 mg/kg, i.p.) were given either 30 min before or 2 h after APAP administration. Administration of $dmPGE_2$ 30 min before APAP significantly improved the survival of animals (Fig. 1A, $p<0.05$). When given 2 h after APAP, $dmPGE_2$ increased the survival of mice, but without statistical significance (Fig. 1A, $p>0.05$). CAY10526 decreased the survival of animals when given either 30 min before or 2 h after APAP, although the differences did not reach statistical significance (Fig. 1B, $p>0.05$ for both comparisons). To determine the plasma ALT concentration, mice were treated as in the previous experiment, except that mice received a lower dose of APAP (150 mg/kg). Fig. 2A and B show mean ALT levels (±SEM) obtained in 8-10 mice per group 20-22 h after APAP administration. Pretreatment or post treatment of mice with $dmPGE_2$ significantly reduced ALT level (Fig. 2A, $p<0.05$ for both comparisons). Fig. 2B shows that CAY10526, if given 2 h after APAP, increased ALT concentration, but, due to high intra group variability, the difference was not significant ($p>0.05$).

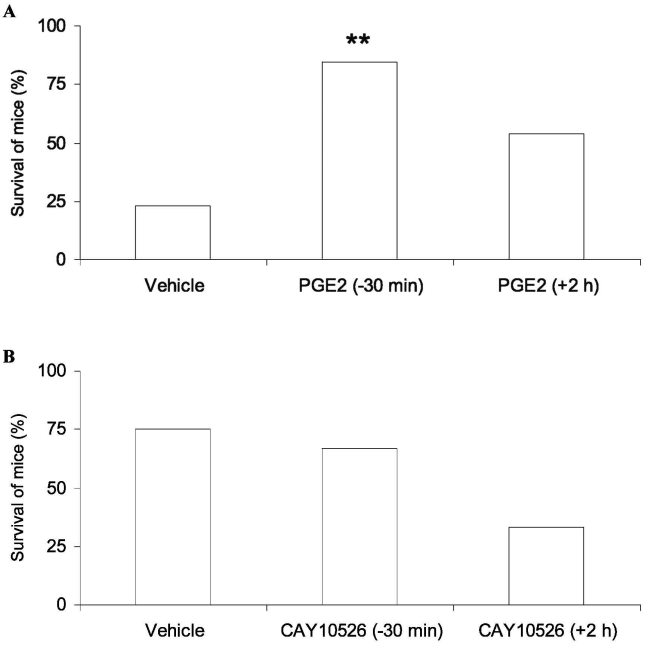

Fig. 1. Influence of $dmPGE_2$ and CAY10526 on survival of mice with APAP-induced liver injury. APAP (300 mg/kg) was given by oral gavage and survival was recorded 48 h later. $dmPGE_2$ (0.2 mg/kg, i.p.) **(A)** and CAY10526 (2.0 mg/kg, i.p.) **(B)** were given either 30 min before or 2 h after APAP administration. Vehicle was given 30 min before APAP administration. Results represent mean ± SEM of 12-13 mice per group. *$p<0.05$ in comparison to vehicle group.

Effect of CAY10526 on ex vivo production of PGE_2

PGE_2 production was determined in supernatants of incubated liver fragments taken from normal mice and mice treated with CAY10526 (2 mg/kg, i.p.) or vehicle 2 h after APAP administration. In comparison to normal (non-treated) mice, treatment with APAP alone (vehicle group) significantly increased production of PGE_2, while treatment with CAY10526 reduced that increase in PGE_2 production (Fig. 3, $p<0.05$ for both comparisons).

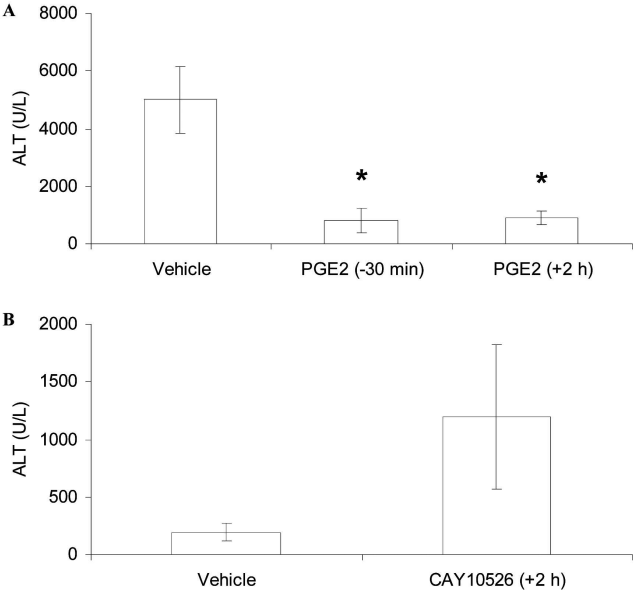

Fig. 2. Influence of $dmPGE_2$ and CAY10526 on plasma ALT concentrations in mice with APAP-induced liver injury. APAP (150 mg/kg) was given by oral gavage and plasma ALT levels were determined 20-22 h later. ALT concentration in normal mice was 28 ± 4 U/L (data not shown). **A.** $dmPGE_2$ (0.2 mg/kg, i.p.) was given either 30 min before or 2 h after, and vehicle 30 min before APAP administration. **B.** CAY10526 (2.0 mg/kg, i.p.) and vehicle were given 2 h after APAP administration. Results represent mean ± SEM of 8-10 mice per group. *$p<0.05$ in comparison to vehicle group.

Liver histology

Macroscopically, the whole liver surface of some APAP treated animals had a mottled appearance; dark red hemorrhagic-necrotic spots were regularly scattered on the yellowish background. Microscopically, the liver damage was graduated using arbitrary scale from 0 to 5 as described in Materials and methods (Fig. 4). The severity of necrosis was quite variable both between animals and also within different parts of the same liver. However, $dmPGE_2$ significantly decreased the number and size of necrotic foci in the liver, which could be easily seen by macroscopic observation and on histological analysis. Macroscopic and microscopic damages of the liver parenchyma appeared more pronounced in mice injected with CAY10526 (Table 1).

Immunohistochemistry to NF-κB p65

Normal (non-treated) mice

NF-κB was expressed in liver cells around blood vessels in Kiernan spaces (afferent arterioles), where numerous small granules were diffusely scattered in the cytoplasm. NF-κB immunostaining was also expressed in individual cells in the lobules and around the central vein (efferent arterioles) (Fig. 5A). Almost all NF-κB immunoreactivity was seen in the cytoplasm and there were only 3.7% positive cells with nuclear positivity to NF-κB (Table 2).

Mice which received APAP alone (vehicle group)

The whole liver tissue was infiltrated with reactive cells, while normal cells were rarely observed. Around blood vessels, there were layers of cells with numerous

Table 1. Effects of $dmPGE_2$ and CAY10526 on APAP-induced liver injury.

			Histopathological scores[c]				
Treatment[a]	0	1	2	3	4	5	%>2[d]
Vehicle + APAP	1	2	2	2	2	1	50
PGE_2 + APAP	6	4	0	0	0	0	0*
APAP + PGE_2	6	3	1	0	0	0	0*
			Histopathological scores[c]				
Treatment[b]	0	1	2	3	4	5	%>2[d]
APAP + vehicle	1	3	2	1	2	0	33
APAP + CAY10526	0	1	1	2	3	2	78

[a]: PGE_2 (0.2 mg/kg, i.p.) was given either 30 min before or 2 h after, and vehicle 30 min before APAP administration. [b]: CAY10526 (2.0 mg/kg, i.p.) and vehicle were given 2 h after APAP administration. Mice were sacrificed and livers were collected 20-22 h after mice received APAP (150 mg/kg). [c]: Histopathological scores were determined and graded by intensity of hepatocellular necrosis from 0 to 5 as described in Materials and Methods. [d]: Scores greater than 2 were considered as significant necrosis. N=9-10 mice per group. *Statistically significant in comparison to vehicle group ($p<0.05$).

vacuoles in cytoplasm and nuclei (Fig. 5B). In the central part of lobules, perimembranous staining pattern and dark colored vacuoles in nuclei were observed. Smaller cells could represent Kupffer cells. Besides cytoplasmic positivity to NF-κB, immunoreactivity was also seen in nuclei (21.8% positive cells) of hepatocytes, indicating significantly higher activity of NF-κB in comparison to normal mice (Table 2, $p<0.001$).

Mice treated with PGE_2

The whole liver tissue was also infiltrated with reactive cells. However, a lot of cells were in the different mitotic periods, which implied liver regeneration. Around the blood vessels, there were reactive cells with small, diffusely scattered granules giving to individual cells a strong positivity in the cytoplasm in comparison to nuclei (Fig. 5C). Immunohistochemical localization of NF-κB, especially around the blood vessels, was mostly seen in the cytoplasm, while its nuclear expression (8.0% cells with NF-κB positive nuclei) was significantly reduced in comparison to mice which received APAP alone (Table 2, $p<0.001$).

Mice treated with CAY10526

Immunohistochemical expression of NF-κB in liver cells showed mostly perimembranous staining pattern. In the central part of lobules, NF-κB was observed in the cytoplasm and in nuclei with vacuoles in both cell compartments (Fig. 5D). Compared to mice which received APAP alone, nuclear positivity to NF-κB in liver cells was higher (23.0% positive cells), but did not reach statistical significance (Table 2, $p>0.05$).

Immunofluorescence to NF-κB p65

Immunofluorescent analysis of NF-κB expression in liver cells generally confirmed previous investigation done by immunohistochemistry. In the group of *normal mice*, only a few cells showed nuclear positivity to (Fig. 6A). Nuclear expression of NF-κB was significantly higher *in mice which received APAP alone* in comparison to normal mice (Fig. 6B), whereas *treatment with PGE_2* reduced that expression (Fig. 6C). In *mice treated with CAY10526*, nuclear positivity to NF-κB revealed a similar pattern as in the group which received APAP alone (Fig. 6D).

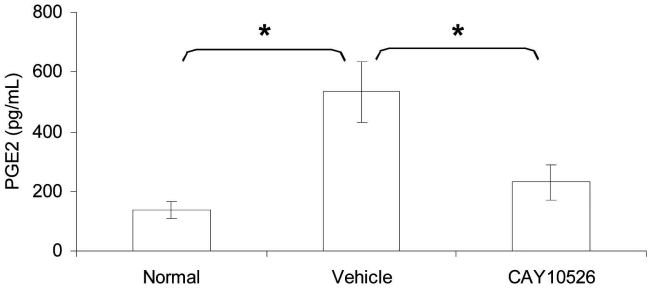

Fig. 3. Effect of CAY10526 on PGE_2 production by the liver fragments. CAY10526 (2.0 mg/kg, i.p.) and vehicle were given 2 h after APAP administration (150 mg/kg). Liver samples were taken 6 h after APAP administration. PGE_2 concentration was determined in supernatants obtained after 1 h incubation of liver fragments. Results represent mean ± SEM of 6 mice per group. *$p<0.05$ in comparison to normal or vehicle group.

Immunohistochemistry to iNOS

Normal mice

Granular accumulations of iNOS were seen in the cytoplasm of hepatocytes organized in small groups, while other hepatocytes were devoid of iNOS reactivity (Fig. 7A). Cells on the inner surface of sinusoid capillaries (endothelial or Kupffer cells) also displayed reactivity to iNOS in their cytoplasm. There were 3.9% cells with cytoplasmic positivity to iNOS (Table 2).

Mice which received APAP alone

iNOS was expressed in the cytoplasm of hepatocytes in the form of small dense granules or large single vacuoles. Some endothelial (or Kupffer) cells expressed iNOS in their cytoplasm as well (Fig. 7B). The number of hepatocytes with cytoplasmic immunoreactivity to iNOS (41.6% positive cells) was significantly higher in comparison to normal mice (Table 2, $p<0.001$).

Mice treated with PGE_2

Granular expression of iNOS was observed in the cytoplasm of hepatocytes, particularly of those situated around the lumen of blood vessels such as central vein (Fig. 7C). The number of liver cells positive to iNOS (20.5% positive cells) was significantly lower in comparison with the group which received APAP alone (Table 2, $p<0.001$).

Mice treated with CAY10526

iNOS was expressed in the cytoplasm of hepatocytes

Table 2. Quantification of immunohistochemistry to NF-κB and iNOS in mice liver cells.

Group	% of liver cells[a] positive to: NF-κB	iNOS
Normal	3.7±0.7	3.9±0.5
Vehicle + APAP	21.8±3.8[b]	41.6±4.4[b]
PGE_2 + APAP	8.0±1.4[b,c]	20.5±4.0[b,c]
APAP + CAY10526	23.0±4.0[b,d]	42.5±4.9[b,d]

[a]: The percentage of positive cells was calculated and expressed as mean ± SEM. [b]: Significantly different from normal mice. [c]: Significantly different from vehicle group. [d]: Significantly different from PGE_2 treated group. Differences were significant at $p<0.001$ level.

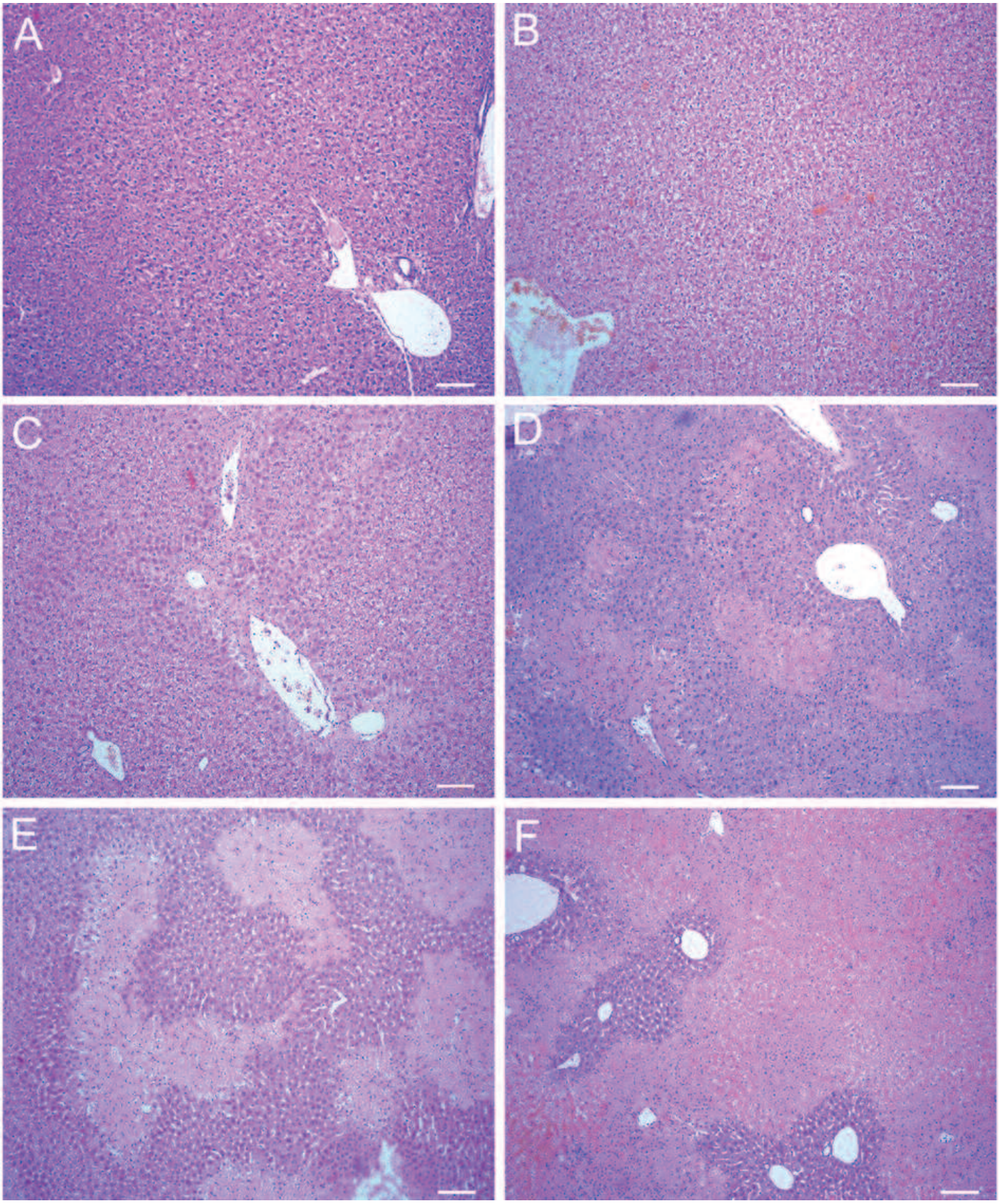

Fig. 4. Histopathological changes in livers from normal and APAP-intoxicated mice. Livers were collected 20-22 h after APAP administration (150 mg/kg). Graduation of liver damage was determined according to the arbitrary scale as follows: degree 0 (**A**), degree 1 (**B**), degree 2 (**C**), degree 3 (**D**), degree 4 (**E)** and degree 5 (**F**). Descriptions of each degree are explained in Materials and Methods. Sections were stained with hematoxylin and eosin (Original magnification, x 100).

in the form of fine granular accumulations or large single vacuoles (Fig. 7D). The number of hepatocytes, which showed positive immunoreactivity to iNOS (42.5% positive cells), was similar as in the group which received APAP alone (Table 2, $p>0.05$).

Effects of PGE_2 and CAY10526 on the plasma concentration of nitrite/nitrate (Fig. 8)

NO synthesis by hepatocytes, measured as a concentration of nitrite/nitrate in mice plasma, was almost undetectable in normal animals. Treatment with APAP significantly elevated nitrite concentration, whereas pretreatment with PGE_2 reduced that level, but not significantly. In CAY10526 treated animals, nitrite level was elevated in comparison to the group which received APAP alone, but again the difference was not significant.

Discussion

Drug toxicity is a severe complication to drug therapy and new drug development. Because APAP is a major cause of acute liver failure in the western world (reviewed in Bernal, 2003; Lee, 2003), we used it as a model of drug-induced liver injury for examination the influence of PGE_2 and its derivatives on drug toxicity mechanisms.

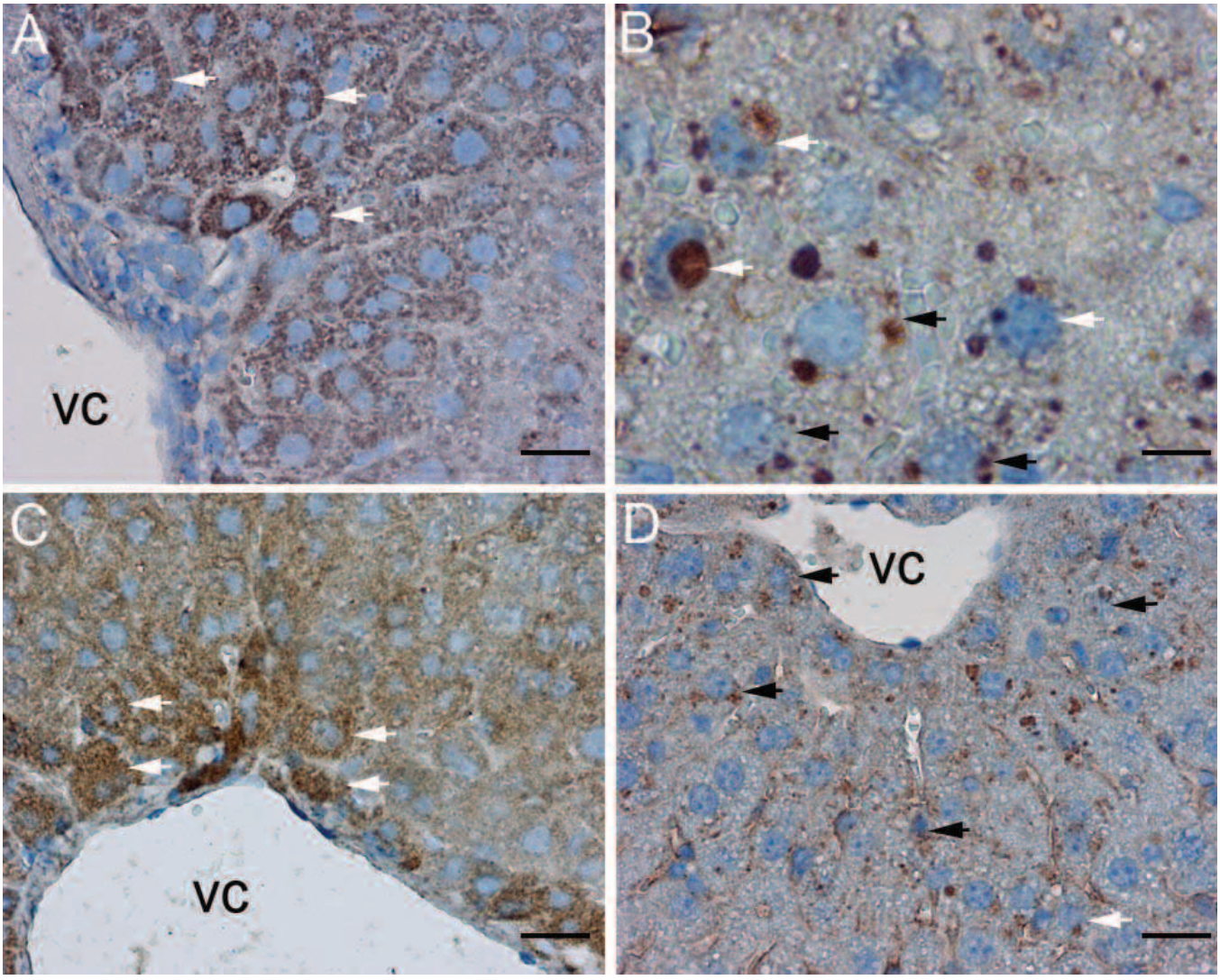

Fig. 5. Immunohistochemical expression of NFκB p65 in mice liver cells. **A**. Normal mice. NF-κB positivity showed mostly diffuse staining pattern within cell cytoplasm (white arrows), particularly in areas close to the central vein. **B.** Mice which received APAP alone (vehicle group). NF-κB staining of liver cells showed perimembranous accumulations like vacuoles both in nuclei (white arrows) and cytoplasm (black arrows). **C.** Mice treated with PGE_2. Expression pattern of NF-κB positive liver cells (white arrows) was similar to that observed as in the group of normal mice (see A). **D.** Mice treated with CAY10526. NF-κB positivity showed a similar pattern of staining as in the group which received APAP alone (see B). NF-κB immunostaining was observed both in nuclei (white arrows) and cytoplasm (black arrows). vc: central vein. A, C, D, x 40; B, x 100.

Although the protective role of PGE_2 and its analogues is well proven in other models of liver injury, especially in those considered as immune system-mediated (reviewed in Quiroga and Prieto, 1993; Muntané et al., 2000; Yin et al., 2007), the protective effect of PGE_2 in a model of APAP-induced toxicity was shown only indirectly. It was reported that a lack of PGE_2 in COX-2 deficient mice (COX-$2^{-/+}$ and $^{-/-}$ mice) and inhibition of COX-2 by selective COX-2 inhibitory drug, celecoxib, increased the hepatotoxic effect of APAP (Reilly et al., 2001). In our previous experiments, we demonstrated that the protective effect of interleukin 1α (IL-1α) on APAP-induced toxicity in mice was abolished by administration of specific anti-PGE_2 antibodies (Renić et al., 1995). The presented results clearly demonstrate that $dmPGE_2$ improves the survival of mice, especially when it was injected into mice before an APAP overdose. Furthermore, hepatic damage, as assessed by plasma ALT concentration and liver histology, was alleviated when animals received $dmPGE_2$ either before or after an APAP administration. These findings are in accordance with previous studies of other authors describing beneficial protective effects of PGE_2 against a variety of hepatotoxic agents other than APAP (ethanol, CCl_4, aflatoxin, Con A, DGalN, LPS alone, or combined with DGalN) (reviewed in Quiroga and Prieto, 1993; Yin et al., 2007). CAY10526 is known as an inhibitor of PGE_2 production through the selective modulation of mPGES-1 expression. In the present experiments, treatment of mice with CAY10526 aggravated the liver damage, as shown by the increase in mortality of animals, elevation of serum ALT level and histopathological changes in liver morphology. As far as we know, this is the first time that CAY10526 has been

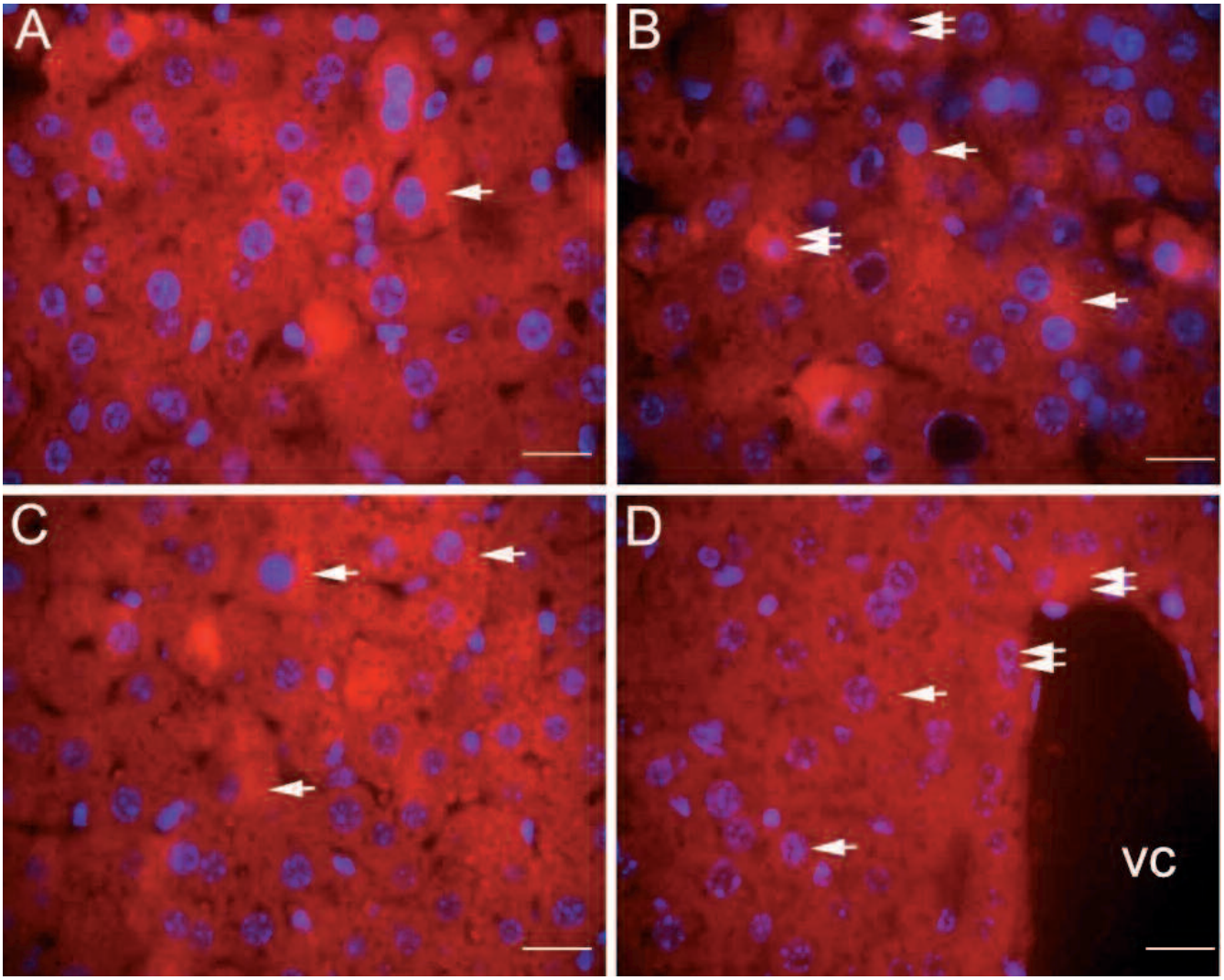

Fig. 6. Immunofluorescence to NF-κB p65 in mice liver cells. **A.** Normal mice. **B.** Mice which received APAP alone. **C.** Mice treated with PGE_2. **D.** Mice treated with CAY10526. White arrows show red colored nuclear or perinuclear positivity to NF-κB. vc: central vein. Scale bar: 10 μm.

used *in vivo* in a model of experimental liver damage induced by a noxious agent. The mechanism of its hepatotoxic action is most probably due to the inhibition of PGE_2 synthesis, because it significantly inhibited ex vivo production of PGE_2 by liver homogenates and it displayed the strongest effect when given to mice 2 h after APAP. This indirectly points to PGE_2 as an endogenously produced hepatoprotective agent, which is supported by our preliminary observation that APAP alone increases synthesis of PGE_2 and PGI_2 in the liver (data not shown).

Our investigations demonstrate that administration of APAP resulted in a higher expression of NF-κB in liver cells from control animals. This is in agreement with previous studies, in which it was shown that APAP-induced liver damage was influenced by oxidative stress (Jaeschke, 2000; James et al., 2003) and that this is dependent, in part. on NF-κB (Bauer et al., 2000; Dambach et al., 2006). Immunohistochemistry and immunofluorescence to NF-κB revealed that the administration of $dmPGE_2$ down regulated in a significant manner the activation of NF-κB and its translocation from the cytoplasm to the nucleus of hepatocytes, while CAY10526 demonstrated mainly the opposite effect. In another animal model, it was shown that selective EP4 agonist suppressed production of proinflammatory cytokines, chemokines, adhesion molecules and NF-κB (Ogawa et al., 2009). In LPS-

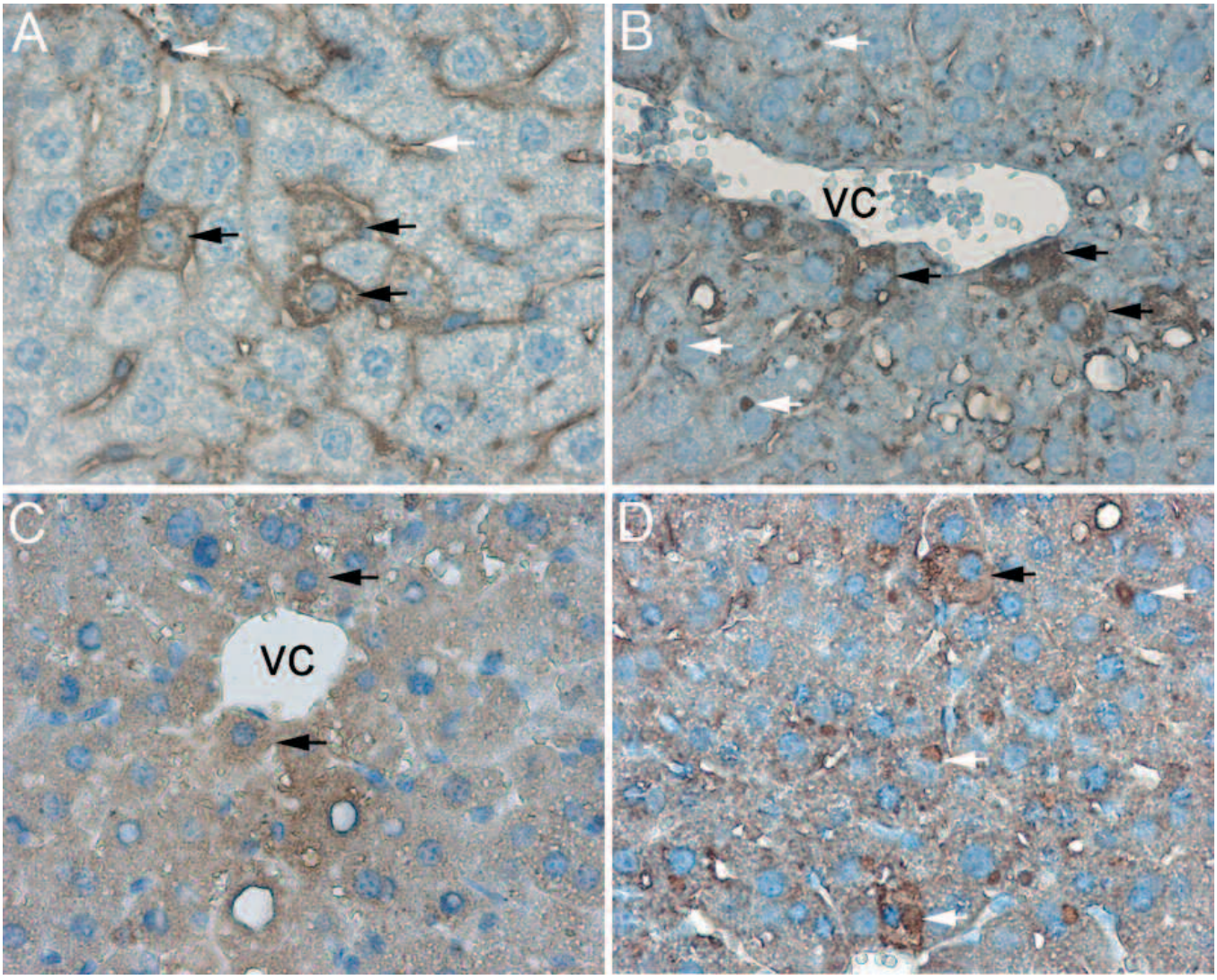

Fig. 7. Immunohistochemical expression of iNOS in mice liver cells. **A.** Normal mice. iNOS positive liver cells showed diffuse granular staining pattern in their cytoplasm (black arrows). iNOS expression was also seen in the cytoplasm of endothelial cells (white arrows). **B.** Mice which received APAP alone. iNOS positivity showed diffuse granular (black arrows) and vacuolar (white arrows) staining pattern in the cytoplasm of hepatocytes. **C.** Mice treated with PGE_2. Diffuse staining pattern of iNOS was seen in the cytoplasm (black arrows) of hepatocytes close to the central vein. **D.** Mice treated with CAY10526. iNOS positivity showed a similar pattern as in the group of mice which received APAP alone (see B), with diffuse granular (black arrows) and vacuolar (white arrows) staining patterns in the hepatocytes. vc: central vein. Scale bar: 25 μm.

treated cultured rat liver macrophages (Kupffer cells), PGE_2 reduced TNF-α production due to down activation of NF-κB (Tran-Thi et al., 1995). NF-κB is known to upregulate expression of genes controlling inflammatory mediators, including iNOS, TNFα, IL-1, IL-10 and COX-$_2$, each of which has been shown to be implicated in APAP-induced hepatotoxicity (Dambach et al., 2006). Although it is not yet clear whether NF-κB plays a positive or negative role in the pathogenesis of liver toxicity induced by APAP, our results just indicate that a protective effect of PGE_2 could be expressed through inhibition of activation of NF-κB.

The present results also showed that treatment with APAP high significantly increased the percentage of iNOS-positive cells and that PGE_2 significantly diminished expression of iNOS in hepatocytes and reduced NO production, which was reflected by lower (albeit statistically not significant) nitrite/nitrate concentration in plasma in comparison to animals receiving APAP alone. CAY10526 showed slightly inverse action on iNOS production. Our results are in agreement with findings provided by others, which reported a significant increase of NO synthesis in APAP toxicity (Hinson et al., 1998; Jaeschke et al., 2003) indicating that increased synthesis of iNOS has a pathogenic role in hepatitis induced by APAP. However, data in the literature on role of iNOS in liver damage, i.e. whether it is a part of regenerative (protective) or toxic (pathogenic) mechanisms, are highly controversial. This was shown in various models of hepatic damage using different experimental designs, including toxic hepatitis induced by APAP (Bourdi et al., 2002; Gardner et al., 2002; Ito et al., 2003), CCl_4 (Morio et al., 2001), immune-mediated hepatitis (Willuweit et al., 2001), etc. Explanation for these conflicting results might be in conception that iNOS is pathogenic and constitutive NOS (cNOS) protective or by observation that the action of NO and its derivatives depends on precise targets on which it is acting and the level of oxidative tissue injury done by superoxide (Laskin et al., 2001). It was shown in this model that NO scavenges superoxide anion to produce peroxynitrite, which then causes protein nitration and tissue injury (Jaeschke 2000; Jaeschke et al., 2003). Despite its controversial role in hepatotoxicity, NO is generally considered as a proinflammatory mediator and our results, at least in part, support that opinion.

It has been shown that the major producers of endogenous PGs in the liver are Kupffer cells and extra hepatic inflammatory cells recruited to liver by chemoatractants (Decker, 1990). APAP-induced hepatotoxicity was paralleled by significant elevation in synthesis of PGI_2, PGE_2 and TXA_2 from liver homogenates or fragments of treated animals (Guarner et al., 1988, our unpublished data). Possible cytoprotective mechanisms of PGE_2 could be, besides the above discussed pathways, due to vasodilatation (by which it can reduce or reverse hepatic vascular congestion), increase in intracellular cAMP level and stimulation of mitogenesis in hepatocytes (reviewed in Quiroga and Prieto, 1993). Data *in vitro* have revealed that cAMP, as well as PGE_2, inhibits the release of potentially hepatotoxic inflammatory cytokines: TNF-α, IL-1 and IFN-γ (Oh-ishi et al., 1996; Schroer et al., 2002). Taken together with our previous investigations, these findings support the view that PGE_2 has a cytoprotective effect and is involved in the defence of the organism to noxious effects of xenobiotics on liver. According to our results, this protection is mediated, at least partially, through down activation of NF-κB and iNOS. Therefore, much remains to be done to elucidate precise mechanisms underlying cytoprotective effects of PGs in acute liver injury.

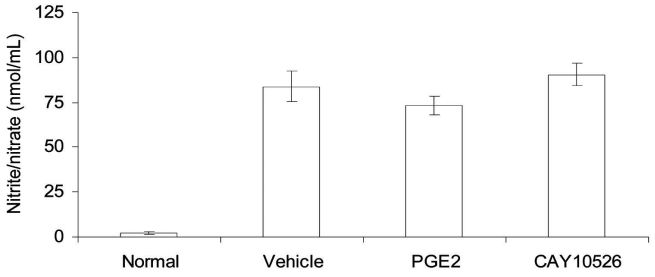

Fig. 8. Effects of PGE_2 and CAY10526 on nitrite/nitrate concentration in mice plasma. APAP (150 mg/kg) was given by oral gavage and plasma nitrite/nitrate levels were determined 6 h later. Nitrite concentration in normal mice was 0.50 ± 0.11 U/L. PGE_2 (0.2 mg/kg, i.p.) and vehicle were given 30 min before, while CAY10526 (2.0 mg/kg, i.p.) was given 2 h after APAP administration. Results represent mean ± SEM of 6 mice per group. Nitrite concentrations in vehicle, PGE_2 and CAY10526 treated groups were significantly higher in comparison to normal mice ($p<0.05$ or better). There were no significant differences between other groups of mice ($p>0.05$).

References

Araki H., Ukawa H., Sugawa Y., Yagi K., Suzuki K. and Takeuchi K. (2000). The roles of prostaglandin E receptor subtypes in the cytoprotective action of prostaglandin E2 in rat stomach. Aliment. Pharmacol. Ther. 14, 116-124.

Bauer I., Vollmar B., Jaeschke H., Rensing H., Kraemer T., Larsen R. and Bauer M. (2000). Transcriptional activation of heme oxygenase-1 and its functional significance in acetaminophen-induced hepatitis and hepatocellular injury in the rat. J. Hepatol. 33, 395-406.

Bernal W. (2003). Intensive care support therapy. Liver. Transpl. 9, 15-17.

Bourdi M., Masubuchi Y., Reilly T.P., Amouzadeh H.R., Martin J.L., George J.W., Shah A.G. and Pohl L.R. (2002). Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. Hepatology 35, 289-298.

Bourne R., Lichtenstein L.M., Melmon K.L., Henney C.S., Weinstein Y. and Shearer G.M. (1974). Modulation of Inflammation and Immunity by Cyclic AMP. Science 184, 119-128.

Cheon H., Rho Y.H., Choi S.J., Lee Y.H., Song G.G., Sohn J., Won N.H. and Ji J.D. (2006). Prostaglandin E2 augments IL-10 signaling and function. J. Immunol. 177, 1092-1100.

Čulo F., Renić M., Sabolović D., Radoš M., Bilić A. and Jagić V. (1995). Ketoconazole inhibits acetaminophen-induced hepatotoxicity in

mice. Eur. J. Gastroenterol. Hepatol. 7, 757-762.

Dambach D.M., Durham S.K., Laskin J.D. and Laskin D.L. (2006). Distinct roles of NF-kappaB p50 in the regulation of acetaminophen-induced inflammatory mediator production and hepatotoxicity. Toxicol. Appl. Pharmacol. 211, 157-165.

Decker K. (1990). Biologically active products of stimulated liver macrophages (Kupffer cells). Eur. J. Biochem. 192, 245-261.

Dey I., Lejeune M. and Chadee K. (2006). Prostaglandin E2 receptor distribution and function in the gastrointestinal tract. Br. J. Pharmacol. 149, 611-623.

Enomoto A., Itoh K., Nagayoshi E., Haruta J., Kimura T., O'Connor T., Harada T. and Yamamoto M. (2001). High Sensitivity of Nrf2 Knockout Mice toAcetaminophen Hepatotoxicity Associated with Decreased Expression of Regulated Drug Metabolizing Enzymes and Antioxidant Genes. Toxicol. Sci. 59, 169–177.

Gardner C.R., Laskin J.D., Dambach D.M., Sacco M., Durham S.K., Bruno M.K., Cohen S.D., Gordon M.K., Gerecke D.R., Zhou P. and Laskin D.L. (2002). Reduced hepatotoxicity of acetaminophen in mice lacking inducible nitric oxide synthase: potential role of tumor necrosis factor-alpha and interleukin-10. Toxicol. Appl. Pharmacol. 184, 27-36.

Guarner F., Boughton-Smith N.K., Blackwell G.J. and Moncada S. (1988). Reduction by prostacyclin of acetaminophen-induced liver toxicity in the mouse. Hepatology 8, 248-253.

Hao C.M. and Breyer M.D. (2008). Physiological regulation of prostaglandins in the kidney. Annu. Rev. Physiol. 70, 357-377.

Harris S.G., Padilla J., Koumas L., Ray D. and Phipps R.P. (2002). Prostaglandins as modulators of immunity. Trends Immunol. 23, 144-150.

Hata A.N. and Breyer R.M. (2004). Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol. Ther. 103, 147-166.

Hinson J.A., Pike S.L., Pumford N.R. and Mayeux P.R. (1998). Nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of acetaminophen in mice. Chem. Res. Toxicol. 11, 604-607.

Ito Y., Abril E.R., Bethea N.W. and McCuskey R.S. (2003). Role of nitric oxide in hepatic microvascular injury elicited by acetaminophen in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 286, 60-67.

Jaeschke H. (2000). Reactive oxygen and mechanisms of inflammatory liver injury. J. Gastroenterol. Hepatol. 15, 718-724.

Jaeschke H., Knight T.R. and Bajt M.L. (2003). The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. Toxicol. Lett. 144, 279-288.

James L.P., Mayeux P.R. and Hinson J. A. (2003). Acetaminophen-induced hepatotoxicity. Drug. Metab. Dispos. 31, 1499-1506.

Janssen-Heininger Y.M., Poynter M.E. and Baeuerle P.A. (2000). Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. Free Radic. Biol. Med. 28, 1317-1327.

Kamanaka Y., Kawabata A., Matsuya H., Taga C., Sekiguchi F. and Kawao N. (2003). Effect of a potent iNOS inhibitor (ONO-1714) on acetaminophen-induced hepatotoxicity in the rat. Life Sci. 74, 793-802.

Laskin J.D., Heck D.E., Gardner C.R. and Laskin D.L. (2001). Prooxidant and antioxidant functions of nitric oxide in liver toxicity. Antioxid. Redox Signal. 3, 261-271.

Lee W.M. (2003). Acute liver failure in the United States. Semin. Liver Dis. 23, 217-226.

Matsuoka T. and Narumiya S. (2007). Prostaglandin receptor signaling in disease. Scientific World J. 7, 1329-1347.

Mazer M. and Perrone J. (2008). Acetaminophen-induced nephrotoxicity: pathophysiology, clinical manifestations, and management. J. Med. Toxicol. 4, 2-6.

Morio L.A., Chiu H., Sprowles K.A., Zhou P., Heck D.E., Gordon M.K. and Laskin D.L. (2001). Distinct roles of tumor necrosis factor-alpha and nitric oxide in acute liver injury induced by carbon tetrachloride in mice. Toxicol. Appl. Pharmacol. 172, 44-51.

Muntané J., Rodríguez F.J., Segado O., Quintero A., Lozano J.M., Siendones E., Pedraza C.A., Delgado M., O'Valle F., García R., Montero J.L., De La Mata M. and Miño G. (2000). TNF-alpha dependent production of inducible nitric oxide is involved in PGE(1) protection against acute liver injury. Gut 47, 553-562.

Nakano H., Monden M., Umeshita K., Murata M., Miyoshi H., Kanai T., Gotoh M. and Mori T. (1994). Cytoprotective effect of prostaglandin I2 analogues on superoxide-induced hepatocyte injury. Surgery 116, 883-889.

O'Connor J.A. and Bennett S. (2005). Acetaminophen toxicity revisited: is drug-induced hepatotoxity immune mediated? J. Pediatr. Gastroenterol. Nutr. 40, 528-529.

Oh-ishi S., Utsunomiya I., Yamamoto T., Komuro Y. and Hara Y. (1996). Effects of prostaglandins and cyclic AMP on cytokine production in rat leukocytes. Eur. J. Pharmacol. 300, 255-259.

Ogawa M., Suzuki J., Kosuge H., Takayama K., Nagai R. and Isobe M. (2009). The mechanism of antiinflammatory effects of prostaglandin E2 receptor 4 activation in murine cardiac transplantation. Transplantation 87, 1645-1653.

Quiroga J. and Prieto J. (1993). Liver cytoprotection by prostaglandins. Pharmacol. Ther. 58, 67-91.

Reilly T.P., Brady J.N., Marchick M.R., Bourdi M., George J.W., Radonovich M.F., Pise-Masison C.A. and Pohl L.R. (2001). A protective role for cyclooxygenase-2 in drug-induced liver injury in mice. Chem. Res. Toxicol. 14, 1620-1628.

Renić M., Čulo F. and Sabolović D. (1995). Protection of acute hepatotoxicity in mice by intrleukin-1a2. Periodicum Biologorum 97, 55-60.

Schroer K., Ying Z., Saunders M.A., Deng W.G., Xu X.M., Meyer-Kirchrath J. and Wu K.K. (2002). Obligatory Role of Cyclic Adenosine Monophosphate Response Element in Cyclooxygenase-2 Promoter Induction and Feedback Regulation by Inflammatory Mediators. Circulation 105, 2760-2765.

Silva V.M., Chen C., Hennig G.E., Whiteley H.E. and Manautou J.E. (2001). Changes in susceptibility to acetaminophen-induced liver injury by the organic anion indocyanine green. Food Chem. Toxicol. 39, 271-278.

Suzuki K., Araki H., Mizoguchi H., Furukawa O. and Takeuchi K. (2001). Prostaglandin E inhibits indomethacin-induced gastric lesions through EP-1 receptors. Digestion 63, 92-101.

Tran-Thi T.A., Decker K. and Baeuerle P.A. (1995). Differential activation of transcription factors NF-kappa B and AP-1 in rat liver macrophages. Hepatology 22, 613-619.

Willuweit A., Sass G., Schöneberg A., Eisel U., Tiegs G. and Clauss M. (2001). Chronic inflammation and protection from acute hepatitis in transgenic mice expressing TNF in endothelial cells. J. Immunol. 167, 3944-3952.

Yin H., Cheng L., Langenbach R. and Ju C. (2007). Prostaglandin I(2) and E(2) mediate the protective effects of cyclooxygenase-2 in a mouse model of immune-mediated liver injury. Hepatology 45, 159-169.

Accepted January 8, 2010