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SCAVENGING PEROXYNITRITE WITH GLUTATHIONE PROMOTES REGENERATION AND ENHANCES SURVIVAL DURING ACETAMINOPHEN-INDUCED LIVER INJURY IN MICE

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Nonstandard Abbreviations:

AAP, acetaminophen; ALT, alanine aminotransferase; BSA, bovine serum albumin; GSH, reduced glutathione; GSSG, glutathione disulfide; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; KPP, potassium phosphate buffer; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NEM, *N*-ethylmaleimide; PCNA, proliferating cell nuclear antigen.

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ABSTRACT

Acetaminophen (AAP) overdose causes formation of peroxynitrite in centrilobular hepatocytes. Treatment with glutathione (GSH) after AAP accelerated recovery of mitochondrial GSH levels, which scavenged peroxynitrite and protected against liver injury at 6 h. The objective of this investigation was to evaluate if GSH treatment has a long-term protective effect against AAP-induced injury and if it promotes liver regeneration. AAP (300 mg/kg) induced severe centrilobular necrosis and increased plasma alanine aminotransferase (ALT) activities (24 h: 3680 ± 320 U/L) in fasted C3Heb/FeJ mice. Only 53% of the animals survived for 24 h. Hepatic glutathione levels were still suppressed by 62% at 24 h compared to untreated controls (19.7 ± 2.6 μmol/g). Glutathione disulfide (GSSG) concentrations were elevated by 455% compared to controls (74 ± 3 nmol/g liver). Treatment with GSH at 1.5 h after AAP treatment attenuated liver necrosis and plasma ALT activities by 62-66% at 24 h. All animals survived **up to 7 days**. The hepatic GSH content recovered to control values, however, the GSSG levels were still elevated **at 48h** (252 ± 26 nmol/g). Expression of proliferating cell nuclear antigen (PCNA) and cell cycle proteins cyclin D_1 and p21, were not detectable in controls or after AAP alone. Treatment with GSH after AAP induced expression of cyclin D_1, p21 and PCNA (12-48h). Thus, GSH treatment after AAP provided long-term hepatoprotection and promotes progression of cell cycle activation in hepatocytes.

Acetaminophen (AAP) is a widely used analgesic and antipyretic. The drug is safe at therapeutic levels but a high dose can cause hepatotoxicity in experimental animals and humans (Nelson, 1990). The mechanism of toxicity involves the metabolic activation of AAP via the P450 system, which results in the formation of an electrophilic reactive metabolite (Jollow *et al.*, 1973), presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin *et al.*, 1984). NAPQI can be conjugated with glutathione (GSH) and excreted. However, after hepatic GSH levels are depleted (Mitchell *et al.*, 1973), NAPQI can covalently bind to cellular proteins (Jollow *et al.*, 1973; Cohen and Khairallah, 1997; Qui *et al.*, 1998). One of the consequences of protein binding is the development of mitochondrial dysfunction with inhibition of respiration (Meyers *et al.*, 1988; Ramsay *et al.*, 1989), a decrease in hepatic ATP levels (Tirmenstein and Nelson, 1990; Jaeschke, 1990), mitochondrial cytochrome c release (Knight and Jaeschke, 2002), mitochondrial oxidant stress and peroxynitrite formation (Jaeschke, 1990; Knight *et al.*, 2001). Recent studies showed significantly reduced AAP-induced liver injury after treatment of rats with an inducible nitric oxide synthase (iNOS) inhibitor (Gardner *et al.*, 1998) and in iNOS gene knock-out mice (Gardner *et al.*, 2002). However, others did not find protection with similar interventions (Michael *et al.*, 2001; Hinson *et al.*, 2002). On the other hand, we demonstrated recently that scavenging peroxynitrite with GSH strongly attenuated hepatic necrosis during the first 6 h after AAP administration (Knight *et al.*, 2002). The protection was observed in the presence of continued mitochondrial dysfunction and oxidant stress. Despite the controversy about the exact source of nitric oxide, these data suggest that peroxynitrite is a critical mediator of AAP toxicity in mice (Knight *et al.*, 2002). However, a potential long-term benefit of peroxynitrite scavenging

has not been demonstrated in this model. Because of the continued mitochondrial oxidant stress in these animals, this is a critical question.

Liver regeneration is a vital process for survival after a toxic insult (Chanda and Mehendale, 1996). Regeneration ensures the replacement of necrotic cells and the full recovery of organ function. Since hepatocytes are mostly in a quiescent state (G_0), the regeneration process requires entry into the highly regulated cell cycle (Fausto, 2000). The first step of this process is the priming of hepatocytes by cytokines such as TNF-α and IL-6 (Akerman *et al.*, 1992; Cressman *et al.*, 1996), which makes cells more responsive to growth factors (Fausto *et al.*, 1995). The exposure to growth factors such as hepatocyte growth factor or transforming growth factor-α results in the expression of cell cycle proteins, e.g., cyclins and cyclin-dependent kinases, and cell cycle inhibitors, e.g., p21 and p27 (Fausto, 2000). The induction of cyclin D_1 is considered a marker of cell cycle progression in hepatocytes (G_1 phase) (Albrecht and Hansen, 1999). Once hepatocytes express cyclin D_1, they have passed the G_1 restriction point and are committed to DNA replication (Fausto, 2000). Interestingly, growth factors also induce inhibitors of the cell cycle, e.g., p21 (Albrecht *et al.*, 1998). The simultaneous expression of activators and repressors of the cell cycle allows a sensitive regulation of liver regeneration (Fausto, 2000).

The aim of our investigation was to evaluate if a treatment regimen with intravenous GSH that mainly scavenges peroxynitrite, has a long-term protective effect against AAP-induced liver injury and enhances survival in this model. Furthermore, we investigated if

the reduced injury and lower mortality is correlated with cell cycle entry (expression of cyclin D_1 and p21) and regeneration.

MATERIALS AND METHODS

Animals. Male C3Heb/FeJ mice with an average weight of 18 to 20 g were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in an environmentally controlled room with 12 h light/dark cycle and free access to food (certified rodent diet no. 8640, Harlan Teklad, Indianapolis, IN) and water. The experimental protocols followed the criteria of University of Arkansas for Medical Sciences and the National Research Council for the care and use of laboratory animals in research. All animals were fasted overnight before the experiments. Animals received an intraperitoneal injection of 300 mg/kg AAP (Sigma Chemical Co., St. Louis, MO) between 8 and 9 am. AAP was dissolved in warm saline (15 mg/ml). Some groups of animals were treated intravenously with 200 mg/kg (0.65 mmol/kg) of GSH between 0 – 3 h after AAP administration. GSH was dissolved in phosphate-buffered saline (25 mg/ml). Other animals were treated s.c. with 450 μg/kg recombinant murine IL-6 (R&D Systems, Minneapolis, MN) 24 h before AAP. All animals were offered food again at 9 h after AAP treatment.

Experimental Protocols. At selected times after AAP treatment, the animals were killed by cervical dislocation. Blood, which was drawn from the vena cava with a syringe, was either added to a heparinized tube and centrifuged to obtain plasma or added to a sterile tube and allowed to clot to obtain serum. Plasma was used for determination of alanine aminotransferase (ALT) activities. Immediately after collecting the blood, the livers were excised and rinsed in saline. A small section from each liver was placed in 10% phosphate buffered formalin to be used in immunohistochemical analysis. A portion of the remaining liver was frozen in liquid

nitrogen and stored at -80° C for later analysis of glutathione and cell cycle protein expression.

Methods. Plasma ALT activities were determined with the test kit DG 159- UV (Sigma Chem. Co., St. Louis, MO) and expressed as IU/liter. Serum levels of interleukin-6 were determined with a murine IL-6 ELISA kit (R&D Systems, Minneapolis, MN). Total soluble GSH and GSSG were measured in the liver homogenate with a modified method of Tietze as described in detail (Jaeschke and Mitchell, 1990). Briefly, the frozen tissue was homogenized on ice in 3% sulfosalicylic acid containing 0.1 mM EDTA. An aliquot of the homogenate was added to 10 mM *N*-ethylmaleimide (NEM) in potassium phosphate buffer (KPP) and another aliquot was added to 0.01 N HCl. The NEM-KPP sample was centrifuged and the supernatant was passed through a C_{18} cartridge to remove free NEM and NEM-GSH adducts (Sep-pak; Waters Associates, Waltham, MA). The HCl sample was centrifuged and the supernatant was diluted with KPP. All samples were assayed using dithionitrobenzoic acid. All data are expressed in GSH-equivalents.

Histology and immunohistochemistry. Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections were cut. Replicate sections were stained with hematoxylin and eosin (H&E) for evaluation of oncotic necrosis (Gujral *et al.*, 2001, 2002). **Necrotic cells were identified using the following morphological criteria: increased eosinophilia, cell swelling, cell lysis, karyorhexis and karyolysis.** The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross section. In general, necrosis was estimated

at low power (x100); questionable areas were evaluated at higher magnification (x200 or x400). The pathologist (A.F.) evaluated all histological sections in a blinded fashion. Sections were also stained for the proliferating cell nuclear antigen (PCNA) using a rabbit polyclonal anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and standard immunohistochemical procedures as described in detail (Chosay *et al.*, 1998).

Western blot analyses. Expression of the cell cycle proteins cyclin D_1 and p21 as well as the proliferating cell nuclear antigen (PCNA) were analyzed by Western blotting as described in detail (Bajt *et al.*, 2000). Briefly, freshly excised liver was homogenized in 25 mM HEPES buffer pH 7.5 containing 5 mM EDTA, 2 mM dithiothreitol, 1% CHAPS, 1 μg/ml pepstatin, leupeptin, and aprotinin. Homogenates were centrifuged at 14,000 g for 20 min at 4^{o}C. Protein concentrations in the cytosolic extracts were determined using the bicinchoninic acid kit (Pierce, Rockford, IL). Cytosolic extracts (50 μg per lane) were resolved by 4-20% SDS-polyacrylamide gel electrophoresis under reducing conditions. Separated proteins were transferred to polyvinylidine difluoride membranes (PVDF, Immobilin-P, Millipore, Bedford, MA) which were then blocked with 5% milk in Tris-buffered saline (TBS: 20 mM Tris, 0.15 M NaCl, pH 7.4) containing 0.1% Tween 20, and 0.1% bovine serum albumin, overnight at 4^{o}C. After washing with TBS, membranes were then incubated with a mouse monoclonal anti-PCNA antibody, a mouse monoclonal anti-cyclin D_1 antibody or a goat polyclonal anti-p21 antibody (all from Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The

membranes were then washed again and incubated with the secondary antibody horseradish peroxidase-coupled anti-mouse or anti-goat IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech. Inc., Piscataway, NJ) according to the manufacturer's instructions.

Statistics. All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way ANOVA followed by Bonferroni *t* test. If the data were not normally distributed, we used the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test. $P < 0.05$ was considered significant.

RESULTS

To investigate a long-term beneficial effect of glutathione treatment against AAP-induced liver injury, animals received an intravenous dose of 200 mg/kg of GSH at 1.5 h after intraperitoneal administration of 300 mg/kg AAP. We previously showed that this dose of AAP induced peroxynitrite formation and caused severe liver injury at 6 h, which was substantially reduced by GSH treatment (Knight *et al.,* 2002). In agreement with these previous data, we observed significant increases in plasma ALT levels and histological evidence of centrilobular necrosis at 6 h after AAP treatment alone (Figure 1A,B). Although the plasma ALT activities did not further increase at later time points (Figure 1A), the area of necrosis expanded to 80% of all hepatocytes at 12-24 h after AAP treatment (Figure 1B). All animals survived up to 12 h. However, only 53% (**8 of 15)** of the AAP-treated mice were alive at 24 h and only 1 **of 15** animals (6%) survived for 48 h. In striking contrast, animals treated with GSH showed significantly less liver injury at all time points up to 48 h as indicated by the significantly lower plasma ALT values and the more than 60% reduction in the area of necrosis (Figure 1). In addition, none of the animals **(0 of 15)** died during the 48 h experiment. **In a separate long-term experiment, all animals treated with AAP + GSH survived for 7 days (5 of 5 animals) with ALT levels not significantly different from untreated animals (100 ± 28 U/L).**

We previously showed that GSH treatment at 1.5 h after AAP did not prevent the mitochondrial oxidant stress 6 h after AAP administration (Knight *et al.,* 2002). Compared to control animals, mice treated with AAP had substantially reduced

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hepatic GSH levels and 4.5-fold higher GSSG concentrations at 24 h after AAP (Figure 2A,B). **This resulted in an increase of the hepatic GSSG-to-GSH ratio from 0.4 ± 0.1 in controls to 6.5 ± 1.2 in AAP-treated mice (P <0.05).** Animals receiving GSH had liver glutathione content similar to untreated controls (Figure 2A) but showed significantly elevated levels of GSSG (Figure 2B). **The GSSG-to-GSH ratio (1.7 ± 0.1) was significantly reduced compared to AAP alone.**

To investigate how the time of treatment with GSH affected the protective effect, animals were treated with GSH at the same time as AAP or at 1.5, 2.25 or 3 h after AAP. Based on plasma ALT activities and histological evaluation of necrosis, the best protection was observed with GSH treatment at the same time as AAP (Figure 3A,B). A significantly reduced injury at 24 h was also found when the animals were treated 1.5 h after AAP. However, neither treatment at 2.25 nor at 3 h had a statistically significant effect on plasma ALT activities (Figure 3A,B). Although not significant, the area of necrosis still showed a trend to reduced injury (Figure 3B). In addition, none of the GSH-treated animals died. This was in contrast to the 50% mortality in animals, which received only AAP.

To investigate if the improved liver injury and survival of GSH-treated animals correlated with evidence of cell cycle activation and regeneration, cyclin D_1 and PCNA levels were analyzed by Western blotting. Cyclin D_1 was not detectable in livers of controls or animals treated only with AAP at 6 – 24 h (Figure 4A). In contrast, cyclin D_1 expression was clearly observed in GSH-treated animals at 12 h, 24 h and 48 h after AAP

administration (Figure 4B). All controls and AAP-treated animals had low hepatic protein levels of PCNA (Figure 5A). In contrast, hepatic PCNA levels progressively increased in GSH-treated animals from 12 h – 48 h after AAP (Figure 5B). Since it is known that cell cycle inhibitors are also activated during regeneration (Albrecht *et al.,* 1998), we evaluated the expression of the cell cycle inhibitor p21. This protein was not expressed in controls and was not up regulated after AAP treatment (Figure 6A). However, in livers of GSH-treated animals, p21 expression was faintly detectable at 6 and 12 h and strongly expressed at 24 and 48 h after AAP (Figure 6B). To assess when GSH has to be administered to induce cell cycle activation, animals were treated with GSH at various times after AAP injection. Analysis for cyclin D_1 expression showed that GSH treatment at the time of AAP administration or at 1.5 h and at 2.25 h after AAP caused cyclin D_1 expression at 24 h (Figure 7). GSH administration at 3 h had no effect.

Although the increased expression of cyclin D_1, p21 and PCNA clearly indicate that liver cells in animals treated with GSH entered the cell cycle, the Western blot analysis did not reveal which cells are affected within the liver lobule. To investigate this issue, liver sections were immunostained for PCNA antigen**.** In agreement with the Western blot analyses, control livers did not stain for PCNA (Figure 8A). Treatment with AAP alone did not increase PCNA immunoreactivity at any time point from 6 – 24 h (Figure 8B and data not shown). In contrast, substantial PCNA staining was observed in hepatocytes surrounding the necrotic areas after treatment with AAP in combination with GSH (1.5 h) for 24 h (Figure 8C).

It is well established that liver regeneration is a multi-step process. Priming of hepatocytes by cytokines such as TNF-α and IL-6 is the initial event (Fausto, 2000). To investigate a potential involvement of IL-6 in the regeneration process of AAP/GSH-treated animals, IL-6 formation was measured. Serum concentrations of IL-6 progressively increased after AAP administration reaching peak levels of almost 400% above baseline at 24 h (Figure 9). In contrast, serum IL-6 values in GSH-treated animals did not significantly increase during the entire 48 h observation period (Figure 9). Since pretreatment with IL-6 can enhance regeneration and protect against ischemia-reperfusion injury (Selzner *et al.*, 1999), we treated the animals with similar doses of murine recombinant IL-6 (450 μg/kg) 24 h before AAP. However, IL-6 treatment had no effect on AAP-induced severe centrilobular necrosis (data not shown).

DISCUSSION

The objective of this investigation was to test the hypothesis that intervention with GSH after acetaminophen treatment has a long-term beneficial effect by reducing cell injury and promoting regeneration for a full recovery and survival. Our previous data showed that treatment with GSH at 1.5 h and later accelerated recovery of mitochondrial GSH levels, attenuated nitrotyrosine formation and reduced liver cell injury up to 6 h after AAP treatment without affecting mitochondrial oxidant stress (Knight *et al.,* 2002). In contrast, GSH treatment at the same time as AAP administration had similar effects but eliminated the mitochondrial oxidant stress. We concluded from these results that injection of GSH at 1.5 h or later minimally affected the detoxification of the reactive metabolite NAPQI but mainly acted as scavenger of peroxynitrite (Knight *et al.,* 2002). This conclusion is supported by the observation that a dose of 400 mg AAP/kg injected i.p. caused maximal protein adduct formation during the first hour with no relevant further increase during the second hour (Roberts *et al.,* 1991). This suggests that under conditions similar to ours, most of the reactive metabolite formation took place during the first hour after AAP administration. However, results by Corcoran *et al.* indicate that after oral doses of 1000 mg AAP/kg maximal covalent binding of the reactive metabolite is observed at 4-6 h (Corcoran *et al.,* 1985a) and the biliary glutathione conjugate excretion is still strong at 4 h (Corcoran *et al.,* 1985b). Although this suggests that reactive metabolite formation may occur later than 1-2 h after AAP, part of the reason for these results is the delayed absorption of an oral dose compared to i.p. injection. In addition, the very high dose of AAP used in these studies may have prolonged the metabolism. However, after i.p. administration of AAP, hepatic glutathione levels are

exhausted in less than 30 min (Jaeschke, 1990; Knight *et al.*, 2001) and covalent binding is maximal between 1-2 h (Roberts, *et al.*,1991). **Intravenously administered GSH is rapidly degraded in the kidney (half-life of plasma glutathione in fasted mice: 5 min) (Jaeschke and Wendel, 1982), the amino acids are re-absorbed in the kidney, the amino acids are re-distributed, taken up into hepatocytes and GSH is re-synthesized. Because of the multiple steps involved, it takes** at least 30-60 min to measure a relevant increase and up to 2 h to fully restore hepatic glutathione levels in starved control animals (Wendel and Jaeschke, 1982). **Because of the delay in treatment and the time needed for de novo GSH synthesis in the liver**, it is most likely that the main impact of GSH administered at 1.5 h after AAP or later was not the detoxification of NAPQI but the scavenging of peroxynitrite **and potentially other reactive oxygen species. It is well documented that GSH and other sulfhydryl-containing compounds such as N-acetylcysteine, are potent scavenger of peroxynitrite, hydrogen peroxide and hypochlorous acid *in vitro* (Liu et al., 1994; Knight et al., 2001; 2002). However, under *in vivo* conditions, both intravenously administered GSH or N-acetylcysteine will stimulate hepatic GSH synthesis by supplying the essential amino acid cysteine.**

The administration of GSH after AAP treatment restored cytosolic and mitochondrial GSH levels to values slightly higher than those of untreated fasted animals at 6 h (Knight *et al.*, 2002). In contrast, animals treated with AAP had still significantly lower levels in both the cytosol and in particular the mitochondria (Knight *et al.*, 2002). This indicated that GSH administration accelerated the recovery of the critical GSH pools at the time

when substantial peroxynitrite was formed in hepatocytes (Knight *et al.*, 2001). Having less liver injury, AAP/GSH-treated animals ate similar to controls when food was offered again at 9 h after AAP administration. Consequently, these animals had supra-physiological levels of hepatic GSH at 24 h. These data are consistent with previous fasting/refeeding experiments (Wendel and Jaeschke, 1988). In contrast, the hepatic GSH levels in AAP-treated animals only recovered to levels of starved animals suggesting that animals with severe liver injury did not eat, which may have contributed to the progressive demise of these animals. Despite the lower GSH levels, GSSG concentrations and the GSSG-to-GSH ratio remained very high in AAP-treated animals. This indicates that there is a continuing oxidant stress in these hepatocytes. We showed previously that the majority of GSSG is located in mitochondria at 6 h (Knight *et al.*, 2001) as well as 24 h (Jaeschke, 1990). Although the GSH-treated animals had elevated GSSG levels, the GSSG-to-GSH ratio was only modestly increased. These data suggest a reduced mitochondrial oxidant stress in AAP/GSH-treated animals at 24 h compared to AAP alone. Thus, the increased oxidant stress in both groups of animals at 6 h got worse in AAP-treated animals but improved in the AAP/GSH-treated animals.

In addition to the reduced oxidant stress and tissue necrosis, all animals treated with GSH showed clear evidence of cell cycle activation and tissue regeneration. In order for hepatocytes to divide (mitosis), they have to leave the resting state (G_0) and go through the cell cycle with DNA synthesis (S-phase) and mitosis (M-phase) (Fausto, 2000). To go through the sequence of these steps, the cell has to pass a number of checkpoints. Expression of cyclin D_1 is a reliable marker that the cell has overcome a restriction point

in G_1 and is irreversibly committed to DNA synthesis (G_1/S transition) (Albrecht and Hansen, 1999). Furthermore, PCNA is synthesized in G_1 and S phase resulting in prominent nuclear staining for this antigen in proliferating cells (Bravo and MacDonald-Bravo, 1987). In addition to promoters of the cell cycle, cell cycle inhibitors are also expressed in the regenerating liver (Albrecht *et al.,* 1998). All three proteins (cyclin D_1, p21 and PCNA) were expressed in livers of AAP/GSH-treated animals as early as 12 h after AAP administration. In contrast, none of these proteins increased in animals treated only with AAP. **Interestingly, cell cycle protein expression at 24 h did not correlate with the severity of the injury at 24 h (Figure 4). In contrast, cell cycle protein expression was observed in livers where peroxynitrite formation was prevented (GSH treatment at t=0) or peroxynitrite was effectively scavenged (GSH treatment at t=1.5 or 2.25 h)** (Knight *et al.*, 2002). Thus, we can conclude that GSH treatment, predominantly by scavenging peroxynitrite, attenuated the progression of AAP-induced liver injury and prevented acute liver failure thereby allowing cell cycle activation and regeneration. This is critical for a full functional recovery of the injured liver and long-term survival.

Hepatocyte regeneration is a multi-step process requiring cytokines and growth factors for its initiation (Fausto *et al.,* 1995). Cytokines prime hepatocytes for the activating effect of growth hormones (Fausto, 2000). Neutralizing TNF-α or eliminating the synthesis of IL-6 prevented a regenerative response after partial hepatectomy (Akerman *et al.*, 1992; Cressman *et al.,* 1996). In addition, pretreatment with recombinant IL-6 promoted liver regeneration in IL-6 gene-deficient mice (Cressman *et al.*, 1996) and

attenuated liver injury during ischemia-reperfusion (Selzner *et al.,* 1999). Interestingly, IL-6 formation was only detectable in AAP-treated animals, which did not show signs of regeneration during the first 24 h. In contrast, GSH treatment eliminated IL-6 formation in AAP-treated mice. Moreover, pretreatment with a high dose of murine rIL-6, similar to previous successful experiments (Cressman *et al.,* 1996; Selzner *et al.,* 1999), had no effect on AAP-induced liver injury. In contrast to these results, Chanda *et al.* (1995) showed that forcing hepatocytes into cell division with a low dose of thioacetamide protected rat livers from subsequent AAP-induced hepatotoxicity. This demonstrates that an early regeneration response can reduce toxic liver injury. On the other hand, treatment with growth factors that stimulate mitosis did not prevent acetaminophen-induced liver failure in dogs (Francavilla *et al.,* 1993). These data suggest that regeneration cannot rescue severely injured cells. Our data agree with these results. The severely damaged hepatocytes after AAP were not able to enter the cell cycle and mount a regenerative response despite the formation of IL-6. However, blunting the progression of the injury by GSH treatment preserved the capacity of the liver to regenerate and therefore to recover from the insult. Interestingly, in contrast to the regenerative response after partial hepatotectomy (Cressman *et al.,* 1996), regeneration after AAP-induced liver injury appears to be independent of IL-6. **One possible explanation for these findings could be that the TNF-α generated in AAP-treated animals may stimulate hepatic regeneration by directly, i.e., independent of IL-6, inducing the expression of transforming growth factor-α (TGF-α), which is a potent mitogen for hepatocytes (Gallucci et al., 2000).**

In summary, our data demonstrated that treatment with GSH after a hepatotoxic dose of AAP attenuated the intracellular oxidant stress and liver cell necrosis and consequently prevented death from liver failure. In contrast to animals, which received AAP alone, all livers of GSH-treated animals showed clear evidence for cell cycle activation and regeneration. We conclude that accelerated restoration of cellular GSH levels at the time of peroxynitrite formation can attenuate the progression of AAP-induced liver injury and allows liver regeneration and ultimately survival.

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FOOTNOTES:

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FIGURE LEGENDS

Figure 1: Plasma ALT activities (A) and hepatocellular necrosis (B) were determined in control C3Heb/FeJ mice and **in animals** 6 - 48 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at 1.5 h after AAP. **The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross section.** Data represent means ± SE of n = 6 animals per group. *P< 0.05 (compared to controls, t = 0). #P<0.05 (compared to AAP)

Figure 2: Liver glutathione content in controls and in animals 24 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at t = 1.5 h after AAP. A. Total glutathione (GSH+GSSG); B. GSSG. All data are given in GSH-equivalents. Data represent means ± SE of n = 6 animals per group. *P< 0.05 (compared to controls, C). #P<0.05 (compared to AAP)

Figure 3: Plasma ALT activities (A) and hepatocellular necrosis (B) were determined in control C3Heb/FeJ mice and **in animals** 24 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (G) at t = 0, 1.5 h, 2.25 h or 3 h after AAP. **The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross section.** Data represent means ± SE of n = 5 animals per group. *P< 0.05 (compared to controls, C). #P<0.05 (compared to AAP)

Figure 4: Western blot analysis of hepatic cyclin D_1 expression in controls and in animals 6 – 48 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at 1.5 h after AAP. Two representative samples are shown per time point. In figure A, a 48 h acetaminophen/GSH (A/G) sample was included as a positive control.

Figure 5: Western blot analysis of proliferating cell nuclear antigen (PCNA) expression in controls and in animals 6 – 48 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at 1.5 h after AAP. Two representative samples are shown per time point. In figure A, a 48 h acetaminophen/GSH (A/G) sample was included as a positive control.

Figure 6: Western blot analysis of hepatic p21 expression in controls and in animals 6 – 48 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at 1.5 h after AAP. Two representative samples are shown per time point. In figure A, a 48 h acetaminophen/GSH (G) sample was included as a positive control.

Figure 7: Western blot analysis of hepatic cyclin D_1 expression in controls and in animals 24 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were

treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at t = 0, 1.5, 2.25 or 3 h after AAP. One or 2 representative samples are shown per time point.

Figure 8: Immunohistochemical staining of liver sections for PCNA in controls (A) and in animals 24 h after treatment with 300 mg/kg acetaminophen (B,C). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at 1.5 h after AAP (C). Necrotic cells in the centrilobular region (*) did not stain for PCNA. PCNA staining was only observed in livers of GSH-treated animals. The most prominent staining was found in the rim of cells between necrotic and healthy tissue (arrows) . CV, central vein; all micrographs X400

Figure 9: Serum interleukin-6 (IL-6) levels were determined by ELISA in control C3Heb/FeJ mice and in animals 6 - 48 h after i.p. injection of acetaminophen (300 mg AAP/kg). Some of the animals were treated with a single i.v. bolus dose of 200 mg/kg glutathione (GSH) at 1.5 h after AAP. Data represent means ± SE of n = 5 animals per group. *$P < 0.05$ (compared to controls, t = 0). $^{\#}P<0.05$ (compared to AAP)

Figure 1A

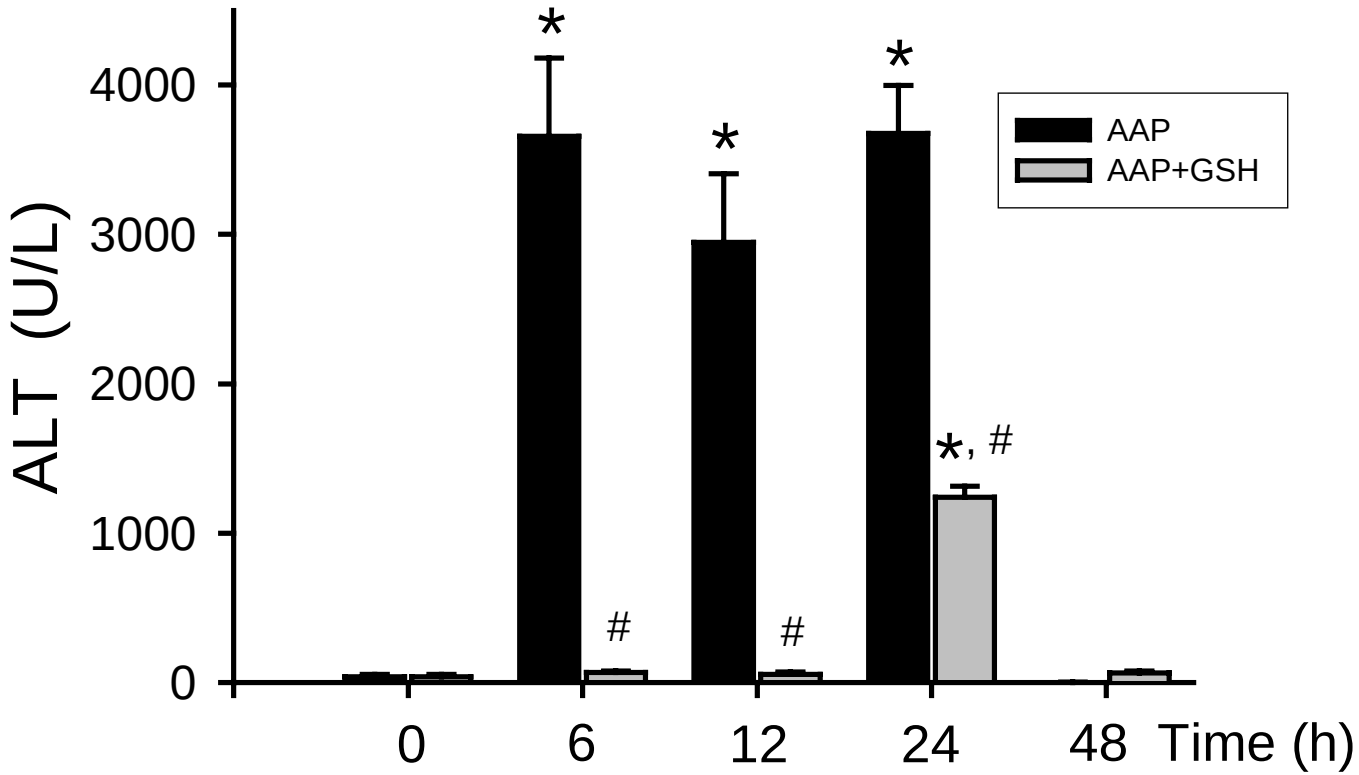
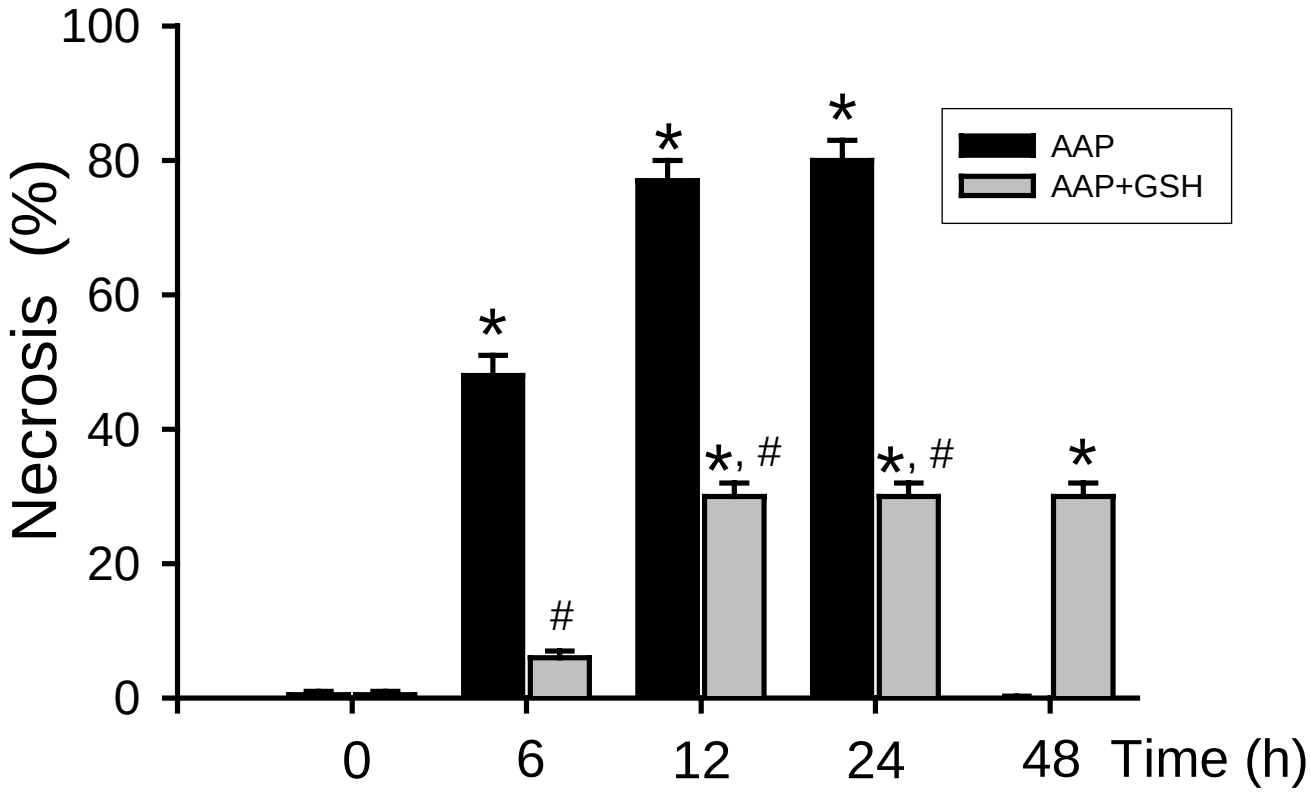


Figure 1B



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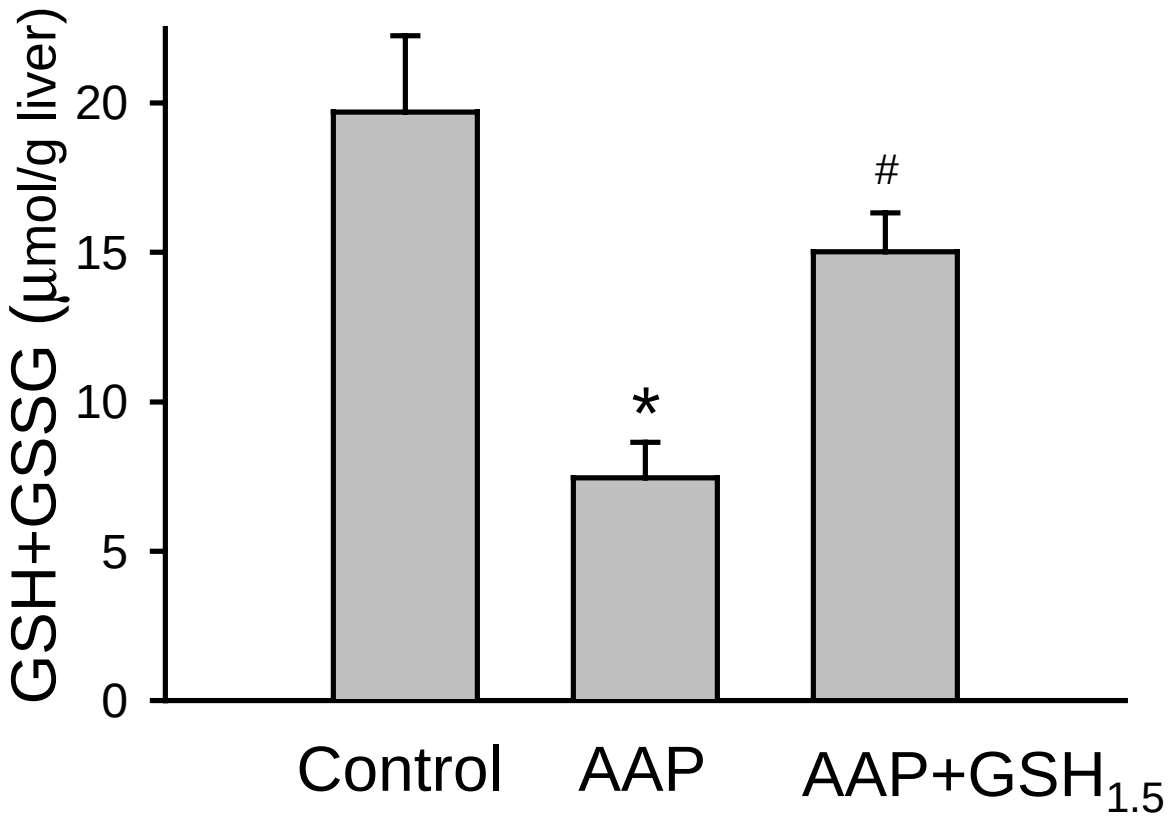

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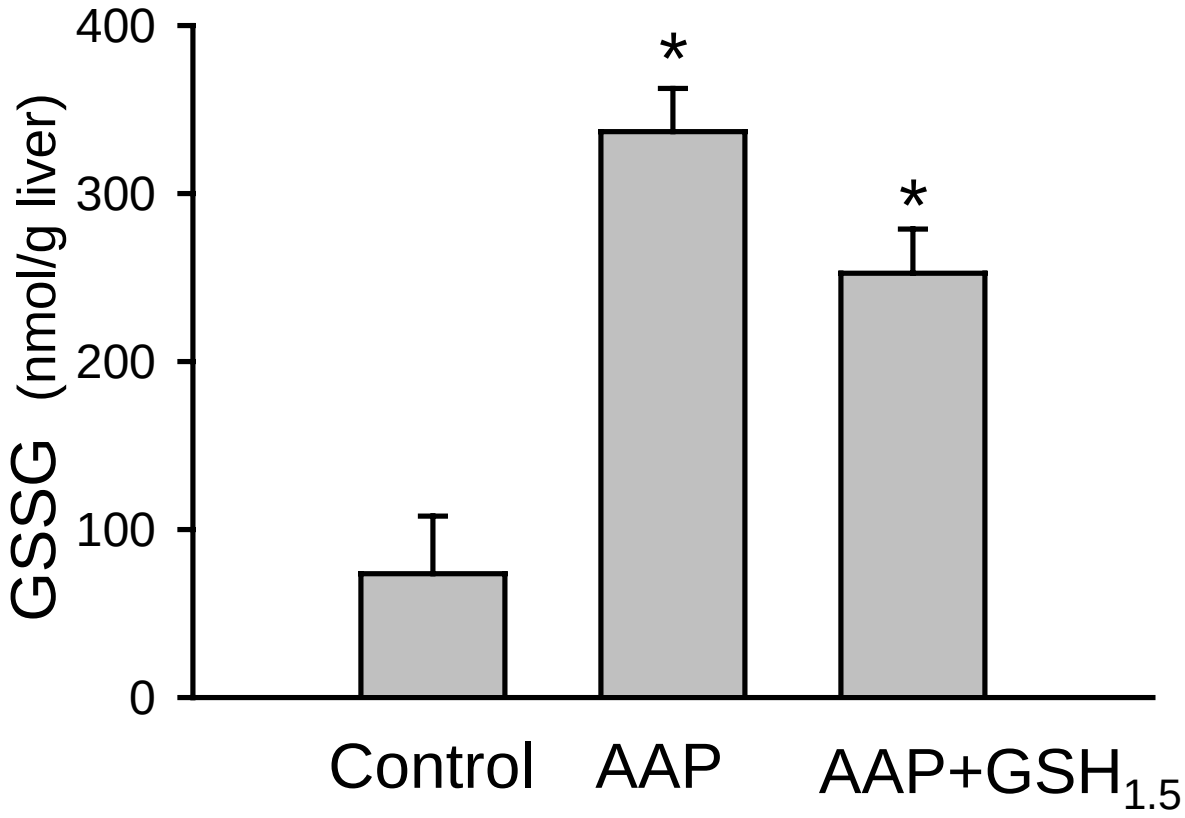

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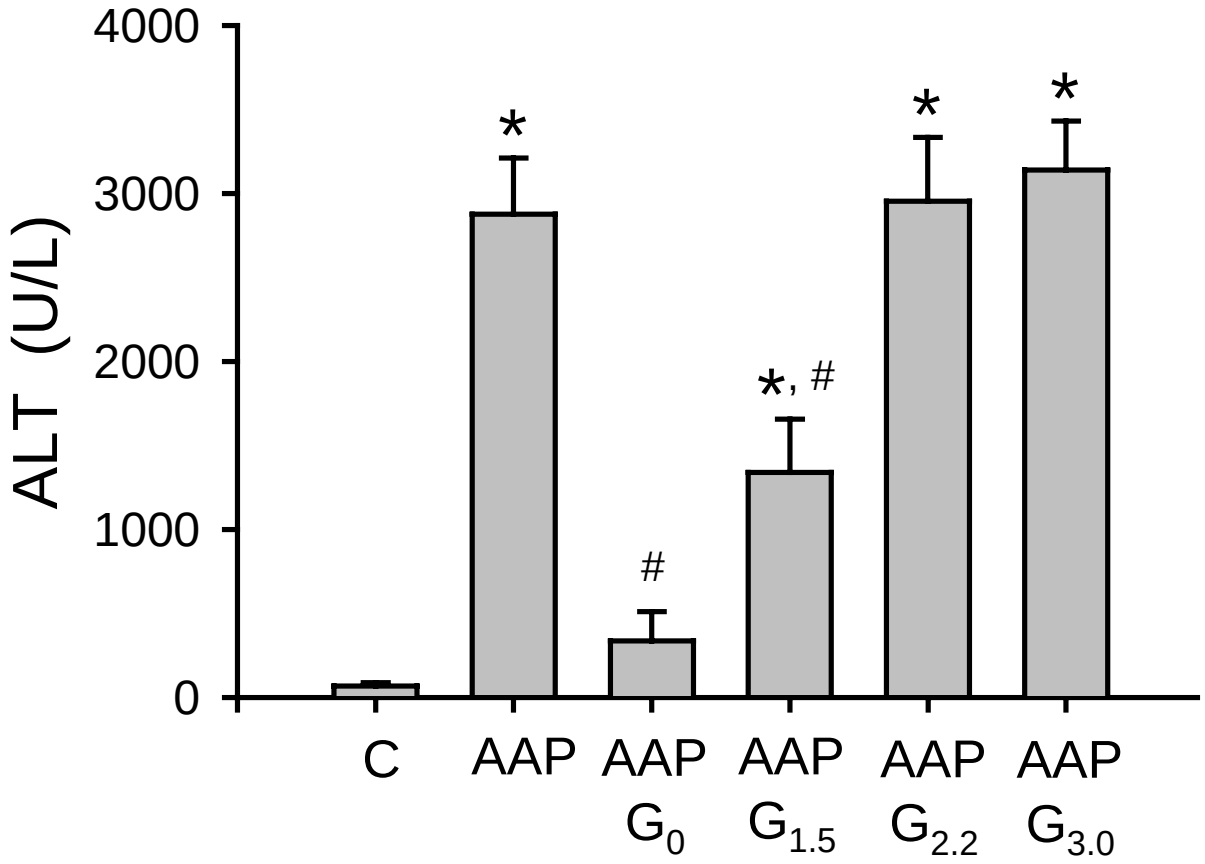

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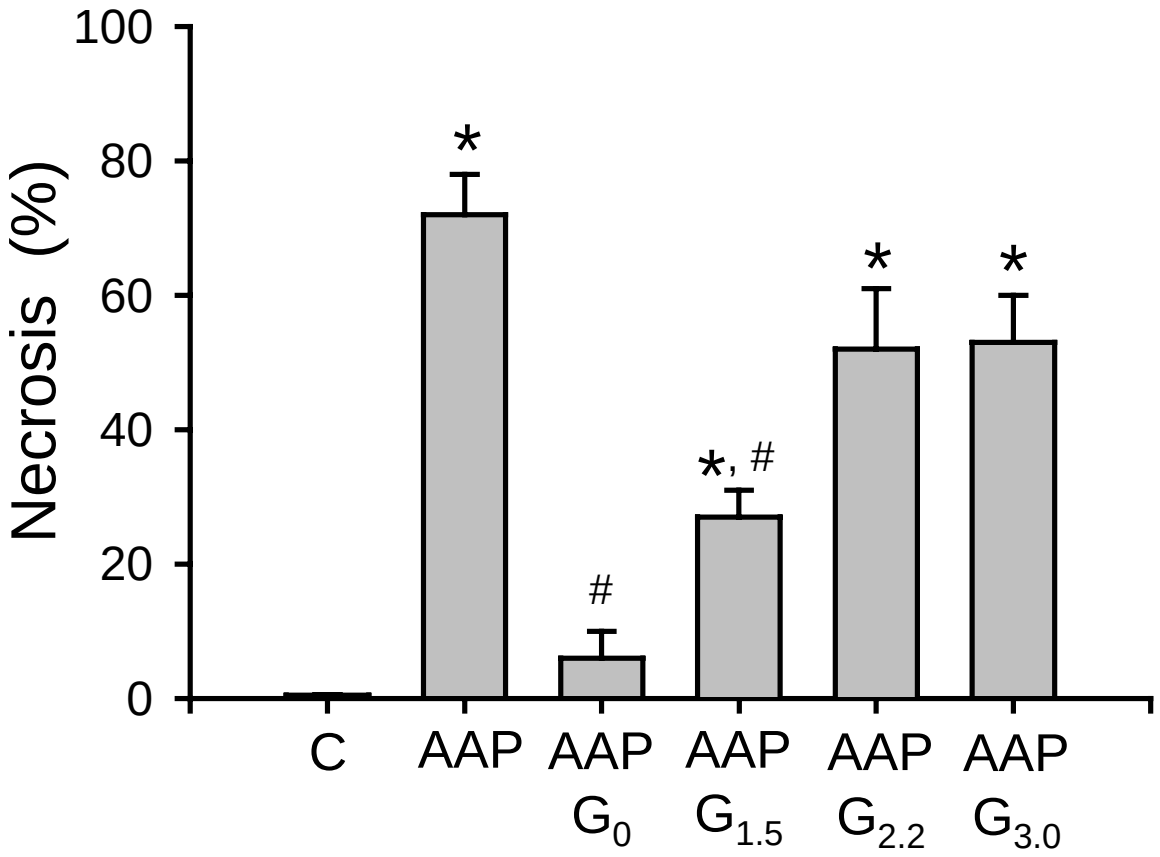

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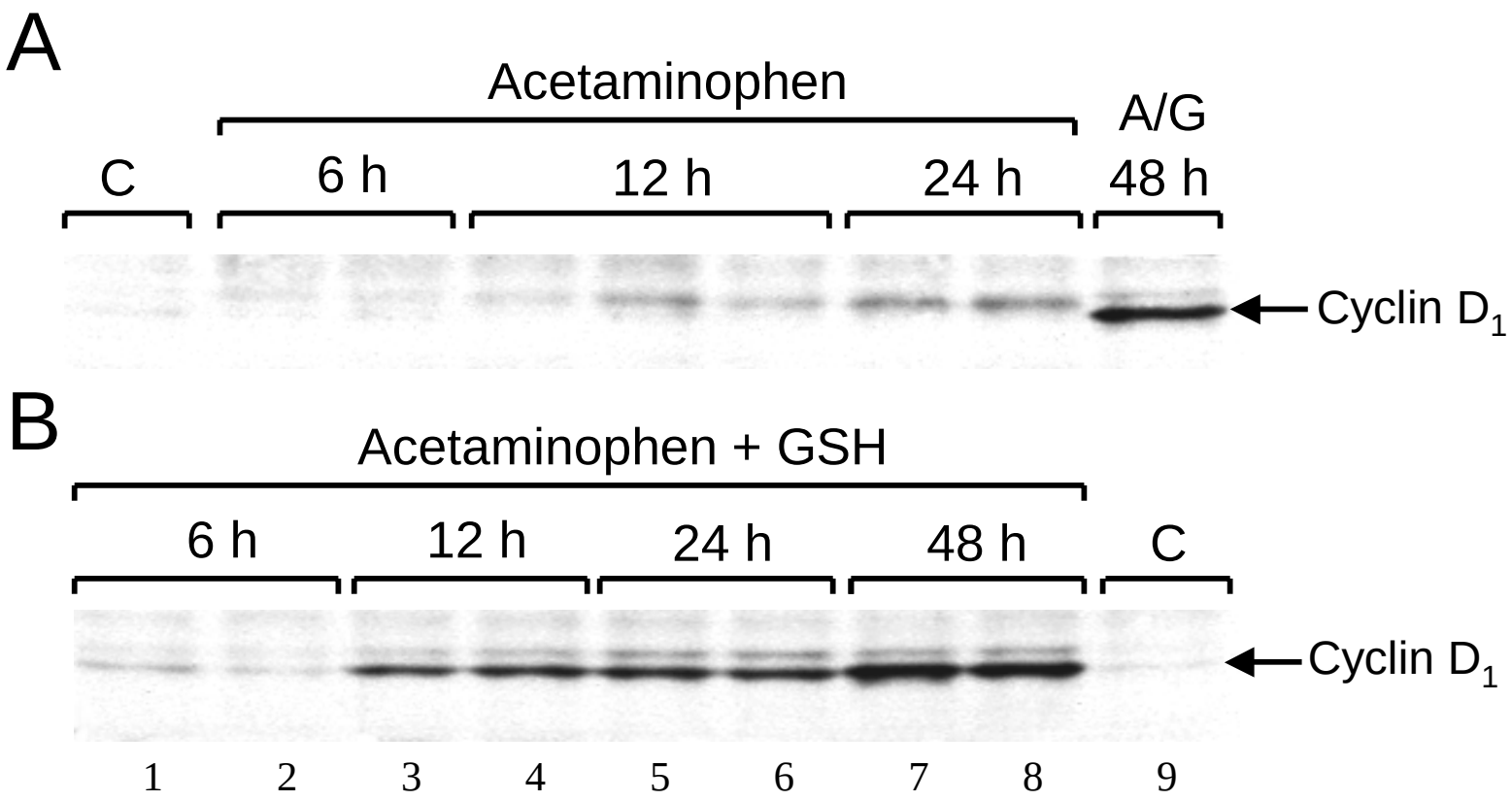

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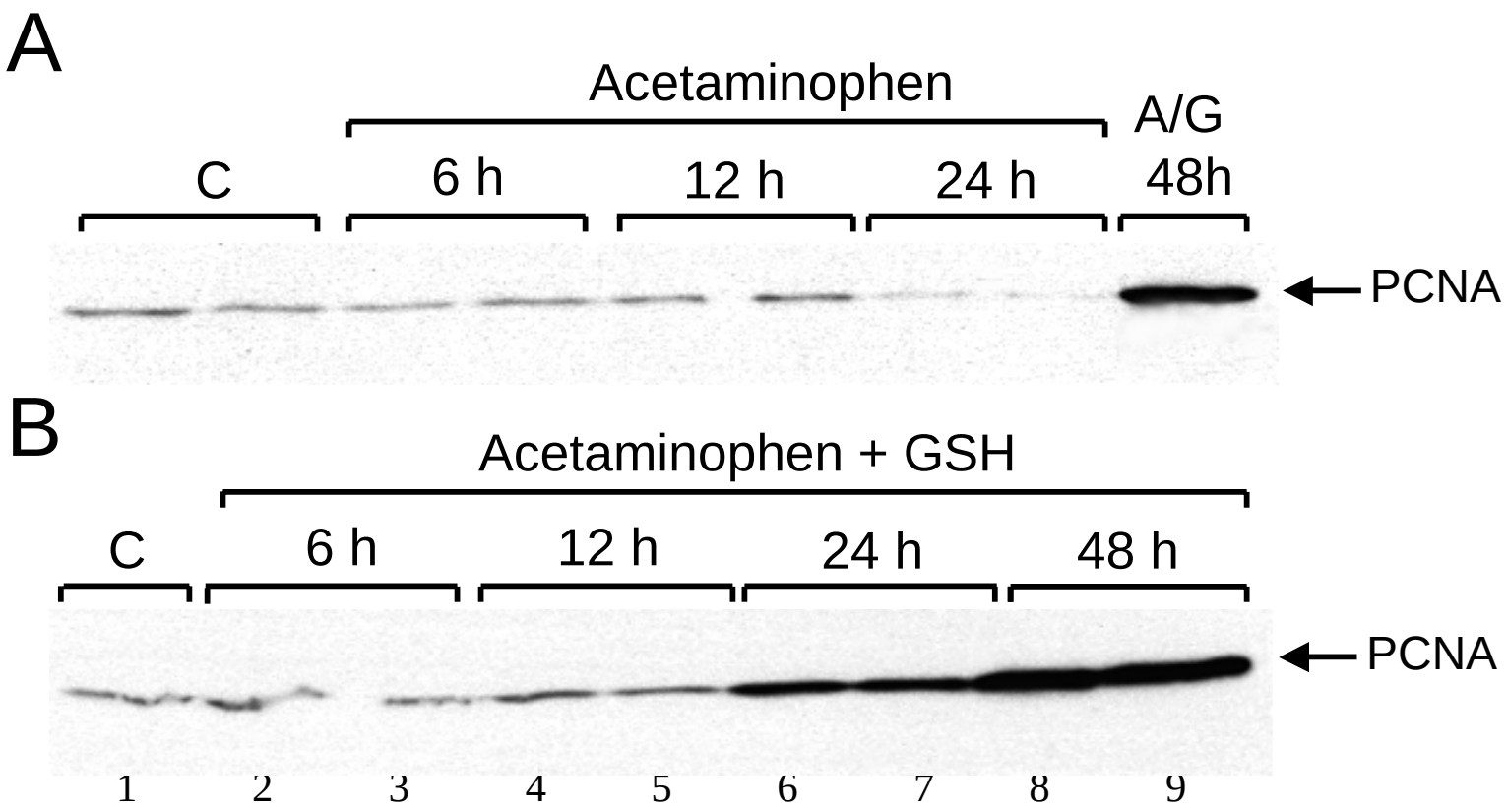

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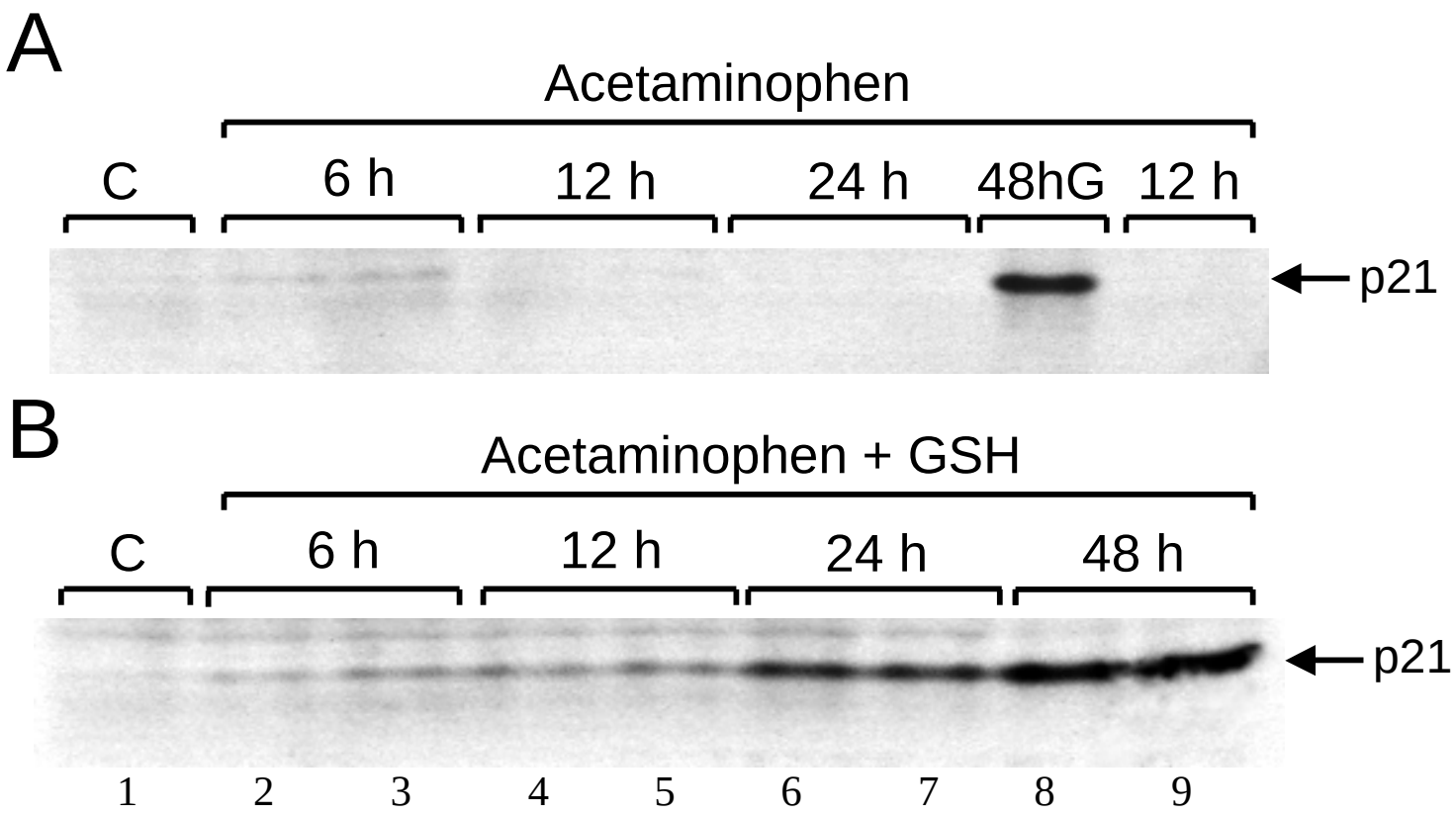

Figure 7

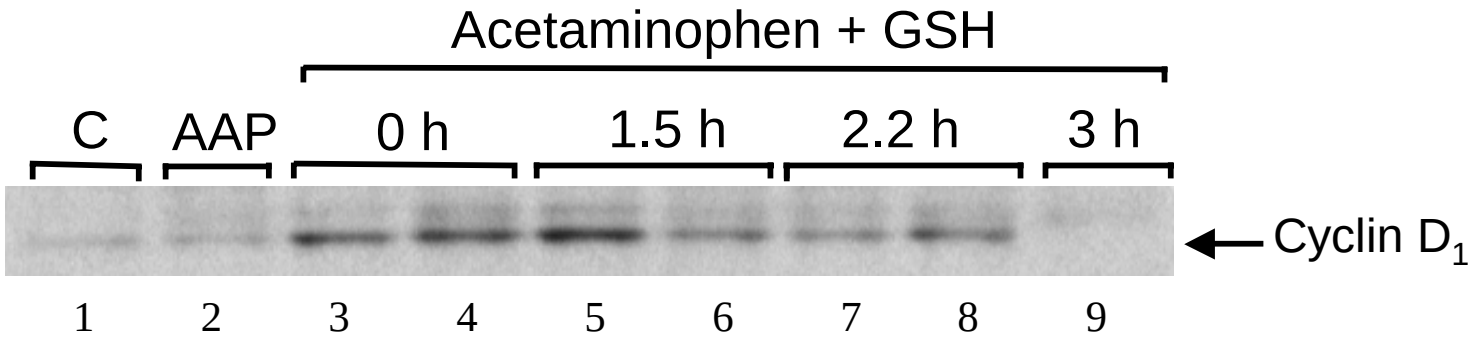

Figure 8

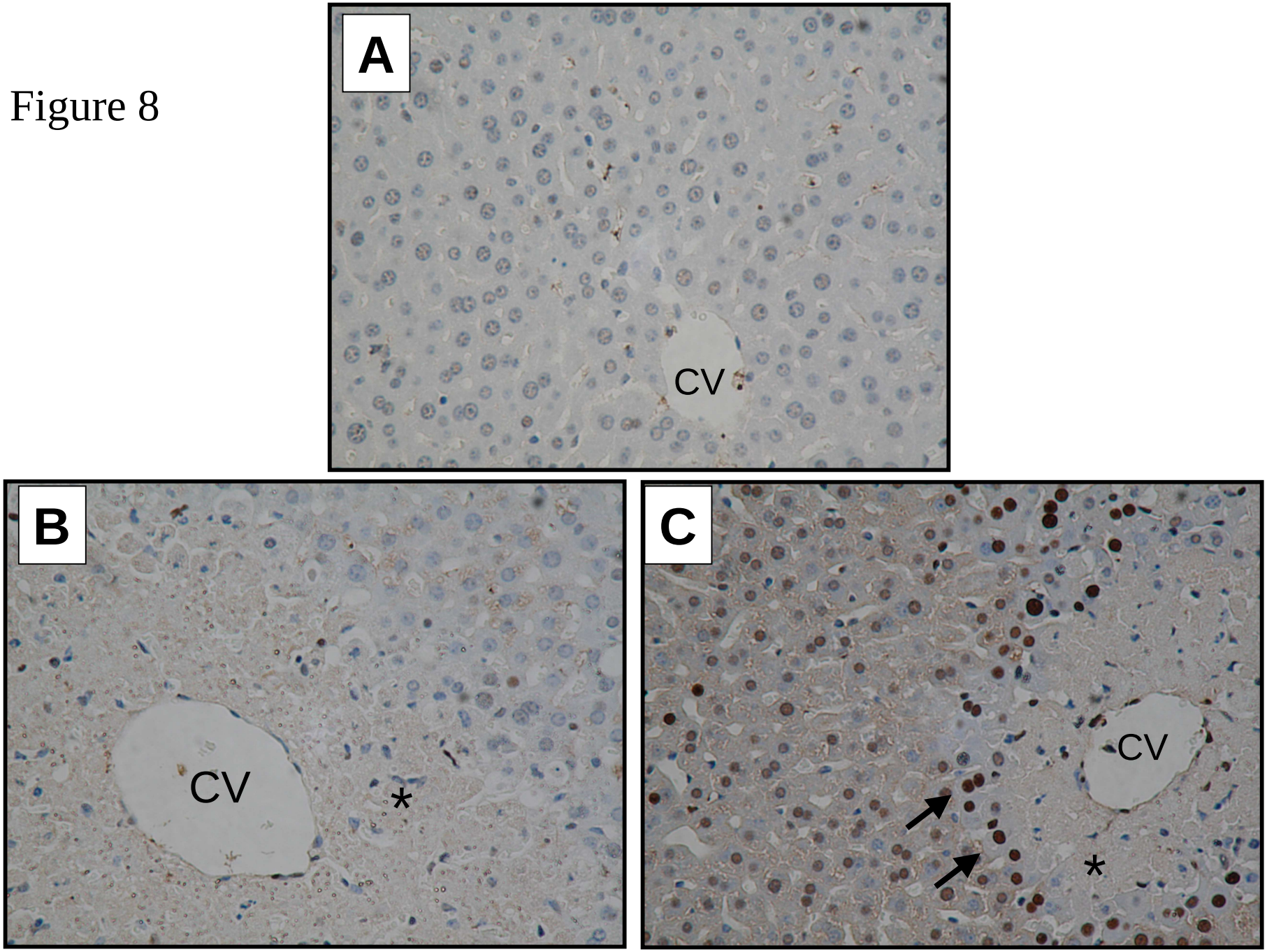

Figure 9