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Title Page

MITOCHONDRIAL BAX TRANSLOCATION ACCELERATES DNA FRAGMENTATION AND CELL NECROSIS IN A MURINE MODEL OF ACETAMINOPHEN HEPATOTOXICITY

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Running Title page

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Nonstandard Abbreviations:

APAP, acetaminophen; AIF, apoptosis inducing factor; ALT, alanine aminotransferase; MPT, mitochondrial membrane permeability transition pore; NAPQI, *N*-acetyl-p-benzoquinone imine; NT, nitrotyrosine; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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ABSTRACT

Mitochondria generate reactive oxygen and peroxynitrite and release endonucleases during acetaminophen (APAP) hepatotoxicity. Since mitochondrial translocation of Bax can initiate these events, we investigated the potential role of Bax in the pathophysiology of hepatic necrosis after 300 mg/kg APAP in fasted C57BL/6 mice. APAP overdose induced Bax translocation from the cytosol to the mitochondria as early as 1 h after APAP injection. At 6 h, there was extensive centrilobular nitrotyrosine staining (indicator for peroxynitrite formation) and nuclear DNA fragmentation. In addition, mitochondrial intermembrane proteins were released into the cytosol. Plasma alanine aminotransferase (ALT) activities of 5610±600 U/L indicated extensive necrotic cell death. On the other hand, Bax gene knock-out (Bax-/-) mice had 80% lower ALT activities, less DNA fragmentation and less intermembrane protein release at 6 h. However, immunohistochemical staining for nitrotyrosine or APAP protein adducts did not show differences between wildtype and Bax-/- mice. In contrast to the early hepatoprotection in Bax-/- mice, plasma ALT activities (7605±480 U/L) and area of necrosis (53±6% of hepatocytes) in wildtype animals was similar to values in Bax-/- mice at 12 h. In addition, there was no difference in DNA fragmentation or nitrotyrosine immunostaining. Conclusion: The rapid mitochondrial Bax translocation after APAP overdose has no effect on peroxynitrite formation but contributes to the mitochondrial release of proteins, which cause nuclear DNA fragmentation. However, the persistent oxidant stress and peroxynitrite formation in mitochondria may eventually trigger the permeability transition pore opening and release intermembrane proteins independent of Bax.

INTRODUCTION

Acetaminophen (APAP) is considered a safe drug at therapeutic doses (Kuffner et al., 2001; Temple et al., 2006). However, an overdose can trigger severe liver injury and acute liver failure. In fact, APAP overdose is currently the most frequent cause of drug-induced liver failure in the US (Larson et al., 2005). Although administration of *N*-acetylcysteine is an effective early intervention against APAP hepatotoxicity, no therapeutic strategies are available that would target the later stages of the injury process. Therefore, a more detailed understanding of the pathophysiology and mechanisms of cell death *in vivo* are necessary to potentially identify novel therapeutic targets, which could be useful to limit cell injury and prevent liver failure.

It is well established that the mechanism of APAP-induced cell injury is initiated by the formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which first depletes glutathione and subsequently covalently binds to cellular proteins (Nelson, 1990). More recent evidence suggest that the covalent protein adduct formation is a critical initiating event, which requires amplification to cause cell death (Jaeschke et al., 2003; Jaeschke and Bajt, 2006). Mitochondrial proteins appear to be the most critical targets of NAPQI for the injury process (Tirmenstein and Nelson, 1989; Qui et al., 2001). This leads to inhibition of the mitochondrial respiration (Meyers et al., 1988; Ramsay et al., 1989), a selective mitochondrial oxidant stress (Jaeschke, 1990), mitochondrial peroxynitrite formation (Cover et al., 2005), and depletion of cellular ATP levels (Jaeschke, 1990; Tirmenstein and Nelson, 1990). The mitochondrial changes and oxidant stress eventually trigger the mitochondrial membrane permeability transition (MPT) pore

opening (Kon et al., 2004) and necrotic cell death *in vivo* (Gujral et al., 2002) and *in vitro* (Nagai et al., 2002; Kon et al., 2004; Bajt et al., 2004). Recent findings also indicate that mitochondria release intermembrane proteins, such as endonuclease G and apoptosis inducing factor (AIF). These proteins translocate to the nucleus and contribute to nuclear DNA fragmentation during APAP-induced cell injury (Bajt et al., 2006). Nuclear DNA degradation is a critical event in the mechanism of cell death (Shen et al., 1992; Napirei et al., 2006; Bajt et al., 2006) and at least in part this process appears to be linked to mitochondrial dysfunction (Cover et al., 2005). On the other hand, the mitochondrial release of cytochrome c (Adams et al., 2001; Knight and Jaeschke, 2002; El-Hassan, 2003) and second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO) (Bajt et al., 2006) are unable to induce caspase activation presumably due to the declining cellular ATP content (Lawson et al., 1999). However, the events leading to the release of these mitochondrial intermembrane proteins during APAP hepatotoxicity have not been identified.

Bax is a member of the Bcl-2 family of proteins (Chao and Korsmeyer, 1998). Bax is mainly located in the cytosol and, upon stimulation, can translocate to the outer mitochondrial membrane (Er et al., 2006). Together with other proapoptotic Bcl-2 family members such as Bad, truncated Bid and Bak, Bax can form pores in the outer membrane triggering the release of intermembrane proteins, e.g., cytochrome c, Smac/DIABLO, AIF and endonuclease G (Er et al., 2006). Mitochondrial Bax translocation in response to APAP exposure *in vivo* has been observed as early as 2 h (Adams et al., 2001; El-Hassan et al., 2003; Jaeschke and Bajt, 2006). However, the functional significance of this event

for APAP-induced hepatotoxicity remained unknown. Therefore, we tested the hypothesis that mitochondrial Bax translocation may be responsible for inducing nuclear DNA fragmentation and cell death in a murine model of APAP overdose.

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MATERIALS AND METHODS

Experimental protocol. C57BL/6J**,** C57BL/6J-*Bax*^{tm1Sjk}mice (Bax gene knock-out mice, Bax-/-) and the respective age-matched wildtype animals from the colony (C57BL/6J background) were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in an environmentally controlled room with 12 h light/dark cycle and allowed free access to food (certified rodent diet no. 8640, Harlan Teklad, Indianapolis, IN) and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee and followed the criteria of University of Arizona 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 APAP (Sigma Chemical Co., St. Louis, MO) between 8 and 9 am. APAP was dissolved in warm saline (15 mg/ml).

At selected times after APAP treatment, animals were killed by cervical dislocation. Blood was drawn from the vena cava into heparinized syringes and centrifuged. The plasma was used for determination of alanine aminotransferase (ALT) activities (assayed with kinetic test kit 68-B, Biotron Diagnostics, Inc., Hernet, CA). Immediately after collecting the blood, the livers were excised and rinsed in saline. A section from each liver was placed in 10% phosphate buffered formalin to be used in immunohistochemical analyses. The remaining liver was either used to isolate subcellular fractions or frozen in liquid nitrogen and stored at -80° C.

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 necrosis (Gujral *et al.*, 2002). 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 nitrotyrosine (NT) protein adducts with the DAKO LSAB Peroxidase Kit (K684) (DAKO Corp., Carpinteria, CA), which was used according to the manufacturer's instructions. The anti-nitrotyrosine antibody was obtained from Molecular Probes (Eugene, OR) (Knight et al., 2002). For the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, sections of liver were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) as described in the manufacturer's instructions (Gujral et al., 2002). Sections were also stained for APAP protein adducts using a rabbit polyclonal anti-acetaminophen antibody (Cell Sciences, Inc., Clanton, MA) and the DAKO LSAB Peroxidase Kit.

Isolation of subcellular fractions and western blotting. Mitochondria and cytosolic fractions were isolated as described (Cover et al., 2005). Briefly, the liver was homogenized in ice cold isolation buffer (pH 7.4) containing 220 mM mannitol, 70 mM sucrose, 2.5 mM HEPES, 10 mM EDTA, 1 mM EGTA, and 0.1% bovine serum albumin. Mitochondria were isolated by differential centrifugation (10,000 g) and washed with 2 ml of isolation buffer. The supernatant of the 10,000g spin was centrifuged at 100,000 g

and the supernatant represented the cytosolic fraction. Purity of cell fractions using this isolation procedures is routinely >95% as measured by LDH activities (cytosol) and succinate dehydrogenase (mitochondria) (Cover et al., 2005). Following the isolation, mitochondrial and cytosolic content of Bax, Smac/Diablo, cytochrome c, AIF and endonuclease G were analyzed by Western blotting as described in detail (Bajt et al., 2000). Briefly, cell fractions were 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 extracts were determined using the bicinchoninic acid kit (Pierce, Rockford, IL). 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 primary antibodies, e.g., a rabbit anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA), an anti-AIF monoclonal antibody (Epitomics, Burlingame, CA), rabbit anti-endonuclease G polyclonal antibody (Chemicon International, Temecula, CA), rabbit anti-cytochrome c polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or an antibody against Smac/DIABLO (BD Biosciences Pharmingen, San Jose, CA) for 2 h at room temperature. The membranes were then washed again and incubated with the secondary antibody horseradish peroxidase-coupled anti-rabbit 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. Data are expressed as means ± S.E. Comparison between two groups were performed with Student's *t*-test or one-way ANOVA followed by Bonferroni *t*-test for multiple groups. If the data were not normally distributed, the Mann-Whitney test was applied for comparison of two groups and the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test for multiple groups. $P<0.05$ was considered significant.

RESULTS

Bax and early APAP-induced liver injury. Treatment of C57BL/6 mice with a moderate overdose of APAP (300 mg/kg) resulted in mitochondrial Bax translocation as early as 1 h after APAP with further increases during the next several hours (Figure 1A). Parallel to the translocation of Bax to the mitochondria there was release of cytochrome c into the cytosol (Figure 1B). Cell injury, indicated by the release of ALT into the plasma, started to increase between 2 and 4 h (Figure 1C). In fact, in a repeat experiment ALT values increased already at 3 h (data not shown), which means that the first cells start to lose viability between 2 and 3 h. This suggests that mitochondrial Bax translocation and release of mitochondrial intermembrane proteins is clearly an event that began before the onset of cell death. To evaluate the pathophysiological relevance of Bax in APAP hepatotoxicity, cell injury was assessed in wildtype (WT) and in Bax gene deficient (Bax-/-) mice 6 h after APAP administration. WT animals had substantial parenchymal cell injury as indicated by the high levels of plasma ALT activities (Figure 2A). In contrast, APAP-induced injury was reduced by 80% in Bax-/- mice (Figure 2A). Western blot analysis of Bax expression in liver mitochondria confirmed the mitochondrial translocation of Bax in wildtype animals at 6 h after APAP administration (Figure 2B). In addition, it showed the complete absence of Bax protein in hepatic mitochondria or cytosol of Bax-/- mice (Figure 2B).

Bax and APAP-induced nuclear DNA damage. Nuclear DNA fragmentation is a hallmark of APAP hepatotoxicity (Ray et al., 1990; Lawson et al., 1999). DNA strandbreaks during APAP-induced liver injury can be visualized by the TUNEL assay

(Lawson et al., 1999; Cover et al., 2005). As shown in a representative tissue section of a WT mouse (ALT activities: 5100 U/L), there is extensive nuclear and cytosolic staining of centrilobular hepatocytes with the TUNEL assay (Figure 3C). This finding demonstrates nuclear DNA strandbreaks and the release of large DNA fragments into the cytosol in these cells (Figure 3C). On the other hand, the number of TUNEL-positive hepatocytes was drastically reduced in Bax-/- animals (ALT activities: 1320 U/L) (Figure 3D).

Bax and the release of mitochondrial intermembrane proteins. Mitochondrial Bax can trigger the release of intermembrane proteins from mitochondria. Since APAP induces the release of endonuclease G and AIF from mitochondria and their translocation to the nucleus (Bajt et al., 2006), we tried to assess if endonuclease G or AIF is detectable in the cytosol 6 h after APAP treatment. Unfortunately, neither endonuclease G nor AIF was detected in the cytosol (data not shown). Therefore, we used cytochrome c and Smac/DIABLO as indicator for mitochondrial protein release (Figure 4). In control animals, there was little cytochrome c or Smac/DIABLO present in the cytosol. However, APAP exposure caused a substantial increase of cytosolic cytochrome c and Smac/DIABLO levels in WT animals but not in Bax-/- mice (Figure 4).

Bax and APAP protein adduct and peroxynitrite formation. It is well established that APAP overdose induces peroxynitrite formation as indicated by the appearance of NT protein adducts in centrilobular hepatocytes (Hinson et al., 1998; Knight et al., 2001). Because of the selective mitochondrial oxidant stress, peroxynitrite formation is also

localized in the mitochondria (Cover et al., 2005). To investigate if mitochondrial Bax translocation induces peroxynitrite formation, NT protein adducts were evaluated in WT and Bax-/- mice (Figure 5). In untreated controls no NT adducts were detected (Figure 5A,B). On the other hand, extensive NT staining of centrilobular hepatocytes was observed at 6 h after APAP administration. However, there was no difference in the extent of NT staining between WT (ALT: 6300 U/L) and Bax-/- (ALT: 1710 U/L) mice (Figure 5 C,D).

APAP toxicity critically depends on covalent binding of NAPQI to cellular proteins (Cohen et al., 1998), which occurs in all cells undergoing necrosis (Roberts et al., 1991). To evaluate if the reduced injury in Bax-/- mice could have been due to reduced protein binding, tissue sections were stained with an antibody against APAP protein adducts (Figure 6). In control livers of WT and Bax-/- mice, no protein adducts were detected (Figure 6 A,B). In contrast, massive centrilobular staining for APAP protein adducts was observed at 6 h after APAP (Figure 6 C,D). However, APAP adduct staining was the same in WT and Bax-/- mice.

Bax and late APAP-induced injury. Although Bax-/- mice showed substantially reduced liver injury at 6 h after APAP treatment, it was a concern that the absence of Bax did not affect NT staining. Given the fact that we previously established a critical role for peroxynitrite in the mechanism of cell death (Knight et al., 2002), it was important to evaluate the injury also at a later time point. Twelve hours after APAP administration WT animals showed severe liver injury as indicated by high levels of plasma ALT

activities and >50% of necrotic hepatocytes (Figure 7). In contrast to the findings at 6 h, Bax-/- mice had similar liver injury as WT animals at 12 h (Figure 7). Likewise, there was no difference in DNA fragmentation (Figure 3E,F) or NT staining (data not shown) between Bax-/- and WT mice at 12 h.

DISCUSSION

The main objective of this study was to evaluate the functional significance of mitochondrial Bax translocation in the pathogenesis of APAP-induced liver cell injury using a Bax-deficient animal model.

Bax-mediated release of mitochondrial intermembrane proteins. Our data confirmed the previously reported low Bax levels in hepatic mitochondria before treatment and the translocation of cytosolic Bax to the mitochondria in response to APAP overdose after 2-4 h (Adams et al., 2001; El-Hassan et al., 2003; Jaeschke and Bajt, 2006). However, our data indicate that Bax translocation starts even earlier as previously shown. Elevated mitochondrial levels of Bax were found at 1 h, i.e. at the time of maximal GSH depletion and protein binding after this dose of APAP (Roberts et al., 1991; Knight et al., 2001). However, Bax translocation occurred before liver injury suggesting that this process could be an important event in the mechanism of cell death. Generally, it is thought that Bax propagates apoptosis by insertion into the outer mitochondrial membrane and formation of pores through oligomerization, which induces the release of pro-apoptotic factors, e.g. cytochrome c, Smac/DIABLO, AIF and endonuclease G, from the intermembrane space (Er et al., 2006). Consistent with this concept, we observed increased levels of cytochrome c in the cytosol parallel to mitochondrial Bax translocation. However, these events preceded necrotic cell death by several hours. Experiments with cultured hepatocytes showed that cell necrosis occurs immediately after the mitochondrial membrane permeability transition (MPT) pore opening and the

loss of the mitochondrial membrane potential (Kon et al., 2004). In addition, inhibition of the MPT pore opening by cyclosporine A does not only prevent APAP-induced cell death *in vitro* (Kon et al., 2004) but also *in vivo* (Masubuchi et al., 2005). These findings support the hypothesis that mitochondrial Bax translocation induces an early release of mitochondrial intermembrane proteins during APAP hepatotoxicity. Currently, it remains unknown what signal triggers mitochondrial Bax translocation. Recent findings indicate a critical role of c-Jun N-terminal kinase (JNK) activation in APAP-induced liver injury (Gunawan et al., 2006). The phosphorylation of 14-3-3, a cytoplasmic anchor of Bax, by JNK has been shown to trigger mitochondrial Bax translocation (Tsuruta et al., 2004). However, more studies are necessary to investigate if JNK or other mechanisms are responsible for Bax activation in hepatocytes.

Our data showed that in the absence of Bax, liver injury was dramatically reduced at 6 h. There was also a dramatic reduction of DNA fragmentation as indicated by the very limited number of TUNEL-positive hepatocytes in Bax-/- mice. Previous studies found no evidence for a relevant activation of caspases during APAP-induced cell death (Lawson et al., 1999; Adams et al., 2001; Gujral et al., 2002; El-Hassan et al., 2003). In addition, caspase inhibitors do not protect (Lawson et al., 1999; Jaeschke et al., 2006). Thus, it is highly unlikely that the traditional DNase responsible for internucleosomal DNA fragmention during apoptosis, i.e., caspase-activated DNase, plays a relevant role in this process. In contrast, we recently showed that nuclear translocation of AIF and endonuclease G correlates with nuclear DNA fragmentation during APAP-induced cell death *in vitro* (Bajt et al., 2006). Other intermembrane proteins, e.g. cytochrome c and

Smac/DIABLO, remained in the cytosol (Bajt et al., 2006). Both events could be inhibited by preventing mitochondrial dysfunction (Bajt et al., 2006). In addition, scavenging peroxynitrite in mitochondria reduced nuclear DNA fragmentation *in vivo* (Cover et al., 2005). Based on these previous findings and the current observation that the release of cytochrome c and Smac/DIABLO was substantially reduced and DNA fragmentation was strongly attenuated in Bax-/- mice, we hypothesize that mitochondrial Bax translocation induced the release and nuclear translocation of AIF and endonuclease G, which contributed to DNA degradation. Since DNA fragmentation is critical for APAP-induced cell death (Shen et al., 1992; Napirei et al., 2006; Bajt et al., 2006), we conclude that mitochondrial Bax translocation accelerates DNA fragmentation and cell death after APAP overdose. Alternatively, the absence of Bax may have delayed the MPT pore opening, which is critical for APAP-induced cell death (Kon et al., 2004). Recombinant Bax is able to induce the MPT pore opening in isolated mitochondria by interacting with the voltage-dependent anion channel (Narita et al., 1998). The effect of Bax on the MPT pore was Ca^{2+}-dependent and could be inhibited by classical MPT inhibitors, e.g. cyclosporine A (Narita et al., 1998). Consistent with this concept, cyclosporine A was shown to attenuate APAP-induced liver cell injury *in vitro* (Kon et al., 2004) and *in vivo* (Masubuchi et al., 2005). However, more studies are needed to elucidate these mechanisms in more detail.

Bax and mitochondrial oxidant stress. APAP overdose induces a selective mitochondrial oxidant stress and peroxynitrite formation as reflected by formation of glutathione disulfide and NT protein adducts, respectively (Cover et al., 2005).

Scavenging peroxynitrite with GSH prevented DNA fragmentation (Cover et al., 2005) and liver cell injury (Knight et al., 2002). These data suggest that mitochondrial peroxynitrite formation is critical for the mechanism of APAP hepatotoxicity *in vivo*. However, despite the reduced cell injury in Bax-/- mice at 6 h, peroxynitrite formation, as indicated by immunostaining for NT protein adducts, was unaffected. This suggests that mitochondrial Bax translocation is not responsible for mitochondrial peroxynitrite generation. On the other hand, the results appear to contradict the previously documented relevance of peroxynitrite in the mechanism of cell death. However, the fact that at 12 h after APAP treatment DNA fragmentation and the extent of cell necrosis was similar in WT and in Bax-/- mice suggested that the injury process in Bax-/- mice caught up with the one in WT animals. Since the MPT pore opening can be induced by oxidant stress, we hypothesize that the continued oxidant stress and peroxynitrite formation in mitochondria may have triggered the MPT in both WT and in Bax-/- mice, leading to mitochondrial swelling, and rupture of the outer membrane with passive release of intermembrane proteins. However, whether peroxynitrite is the actual cause of the MPT pore opening remains to be investigated *in vivo*. Nevertheless, our data suggest that mitochondrial Bax translocation is responsible for the early nuclear DNA fragmentation, presumably through release of AIF and endonuclease G (Bajt et al., 2006). However, the continued nitrosative stress may override the effect of Bax at later times (>6 h) and cause DNA fragmentation independent of Bax. Thus, mitochondrial Bax translocation accelerates nuclear DNA fragmentation and necrotic cell death during APAP hepatotoxicity. However, deletion of Bax does not afford a permanent protection *in vivo*. Only

scavenging of mitochondrial peroxynitrite results in long-term reduction of liver injury (Knight et al., 2002; Bajt et al., 2003).

Bax and the mode of APAP-induced cell death. Mitochondrial Bax translocation and release of cytochrome c are considered hallmarks of the mitochondrial pathway of apoptosis and is has been suggested that APAP induces apoptosis, which rapidly deteriorates into secondary necrosis (El-Hassan et al., 2003). However, there is no morphological evidence of apoptosis in >90% of dying hepatocytes (Gujral et al., 2002; Napirei et al., 2006), there is no relevant activation of caspases (Lawson et al., 1999; Adams et al., 2001; El-Hassan et al., 2003) and potent pancaspase inhibitors do not protect (Lawson et al., 1999; Jaeschke et al., 2006). In fact, APAP-induced cell injury actually inhibits Fas-mediated apoptosis in hepatocytes due to impairment of mitochondrial function by APAP (Knight and Jaeschke, 2002). There are certain similarities in nuclear DNA fragmentation between APAP-mediated cell death and apoptosis, i.e. formation of small internucleosomal DNA fragments ("DNA Ladder") (Ray et al., 1990; Cover et al., 2005). However, there are other major qualitative and quantitative differences in nuclear DNA damage (Cover et al., 2005). Most importantly, DNA fragmentation during APAP-induced cell death is inhibited by scavenging mitochondrial peroxynitrite (Cover et al., 2005), and prevention of mitochondrial release of intermembrane proteins (Figure 3) but not by inhibitors of caspases (Lawson et al., 1999; Jaeschke et al., 2006). These data together strongly support the conclusion that APAP-induced cell death is caused by oncotic necrosis. However, in contrast to earlier assumptions that oncotic necrosis is triggered by a single catastrophic event leading to

immediate cell death, our data suggest that the cellular stress caused by a chemical such as APAP leads to cellular dysfunction, which needs to be amplified and propagated through signaling mechanisms within the cell to result in oncotic necrosis. The current study provides further evidence that common pathways can be used in apoptotic and oncotic necrotic cell death (Jaeschke and Lemasters, 2003)..

In summary, early Bax translocation has no effect on the mitochondrial oxidant stress and peroxynitrite formation but contributes to the release of proteins from the mitochondrial intermembrane space. Some of these proteins (endonuclease G, AIF) can translocate to the nucleus and cause DNA fragmentation. However, the persistent oxidant stress and peroxynitrite formation in mitochondria may eventually trigger the MPT and release more intermembrane proteins independent of Bax. Thus, mitochondrial Bax translocation is an important early mechanism initiating DNA fragmentation and cell necrosis. However, this mechanism is overwhelmed by the continuous mitochondrial oxidant stress and peroxynitrite formation at later time points.

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FOOTNOTES

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Legends for Figures

Figure 1: Time course of mitochondrial Bax translocation (A) and release of cytochrome c into the cytosol (B) in response to acetaminophen treatment (300 mg/kg). Mitochondria and cytosol were isolated from the livers of 2 animals per group. Plasma alanine aminotransferase (ALT) activities were measured as indicator of acetaminophen-induced liver injury (C). Data represents means ± SE of n = 4 animals per time point.

Figure 2: A. Plasma alanine aminotransferase (ALT) activities as indicator for acetaminophen (APAP)-induced liver injury were measured in C57BL/6 wildtype (WT) and in Bax gene-deficient (Bax-/-) mice 6 h after administration 300 mg/kg APAP. Data represent means ± SE of n = 7 animals per group. *P<0.05 (compared to WT). B. Bax protein expression in hepatic mitochondrial fractions and the cytosol of WT and Bax-/- mice was measured by western blotting. One untreated control of each group is compared to 3 animals treated with APAP for 6 h.

Figure 3: DNA fragmentation was assessed by the TUNEL assay in C57BL/6 wildtype (A,C,E) and in Bax gene-deficient mice (B,D,F). Samples were taken from control animals (A,B), or from mice treated with 300 mg/kg acetaminophen for 6 h (C,D) and 12 h (E,F). pp, periportal; cv, central vein.

Figure 4: Release of the mitochondrial intermembrane proteins cytochrome c and Smac/DIABLO into the cytosol in controls (C) or 6 h after treatment with 300 mg/kg

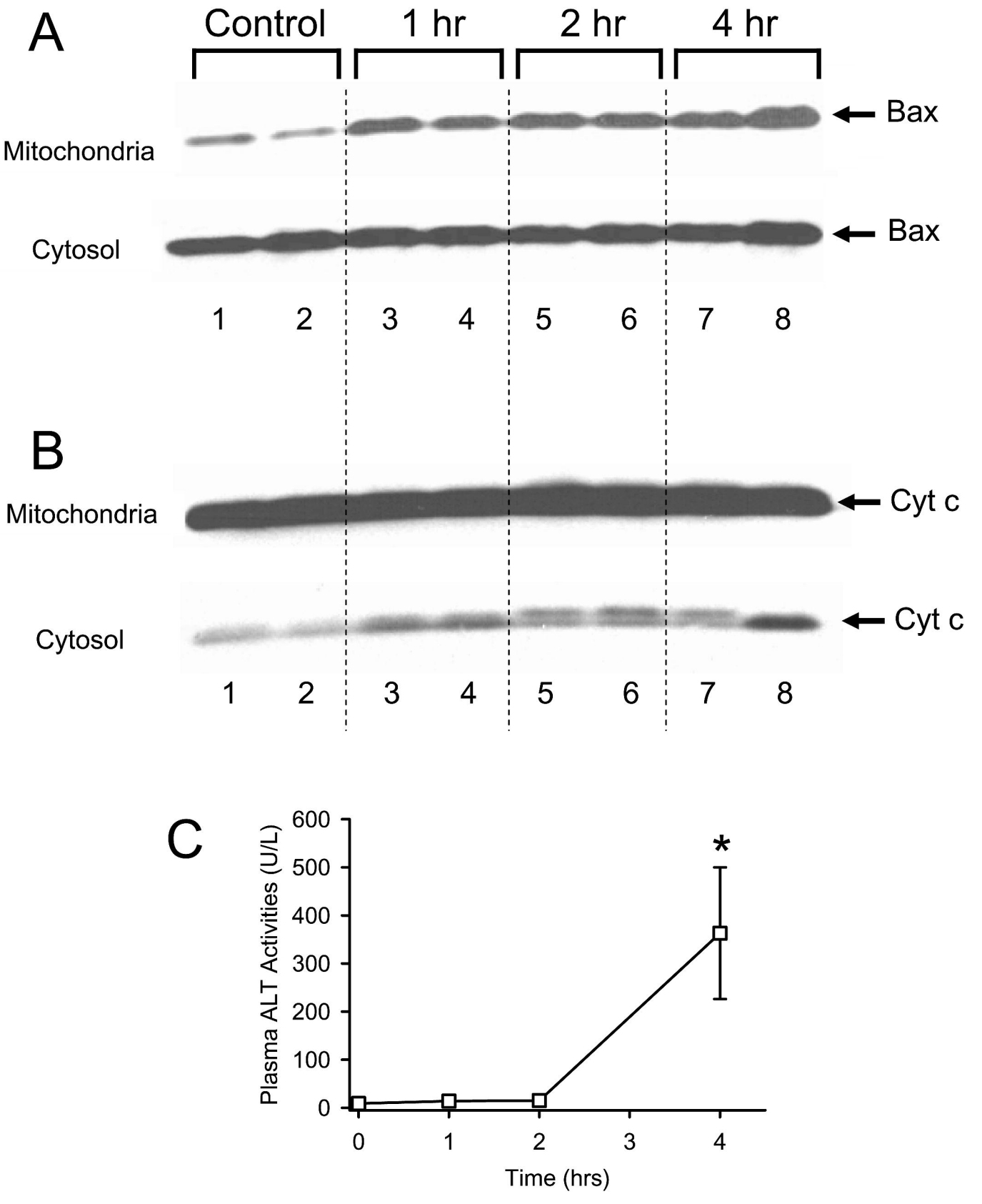
acetaminophen. Cytosol was isolated from 2 animals per group of C57BL/6 wildtype and Bax gene-deficient (Bax-/-) mice.

Figure 5: Liver sections from wildtype (A,C) and Bax gene-deficient mice (B,D) were stained for nitrotyrosine protein adducts. Samples were taken from control animals (A,B), or from mice treated with 300 mg/kg acetaminophen for 6 h (C,D).

Figure 6: Liver sections from wildtype (A,C) and Bax gene-deficient mice (B,D) were stained for acetaminophen protein adducts. Samples were taken from control animals (A,B), or from mice treated with 300 mg/kg acetaminophen for 6 h (C,D).

Figure 7: Plasma alanine aminotransferase (ALT) activities (A) and the area of necrosis (B) were determined as indicator for liver injury in wildtype (WT) and in Bax gene-deficient (Bax-/-) mice 12 h after administration 300 mg/kg acetaminophen. Data represent means ± SE of n = 4 animals per group. *$P<0.05$ (compared to WT).

Figure 1: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Cyt c
1 2 3 4 5 6 7 8
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0 1 2 3 4
Time (hrs)

Figure 2:

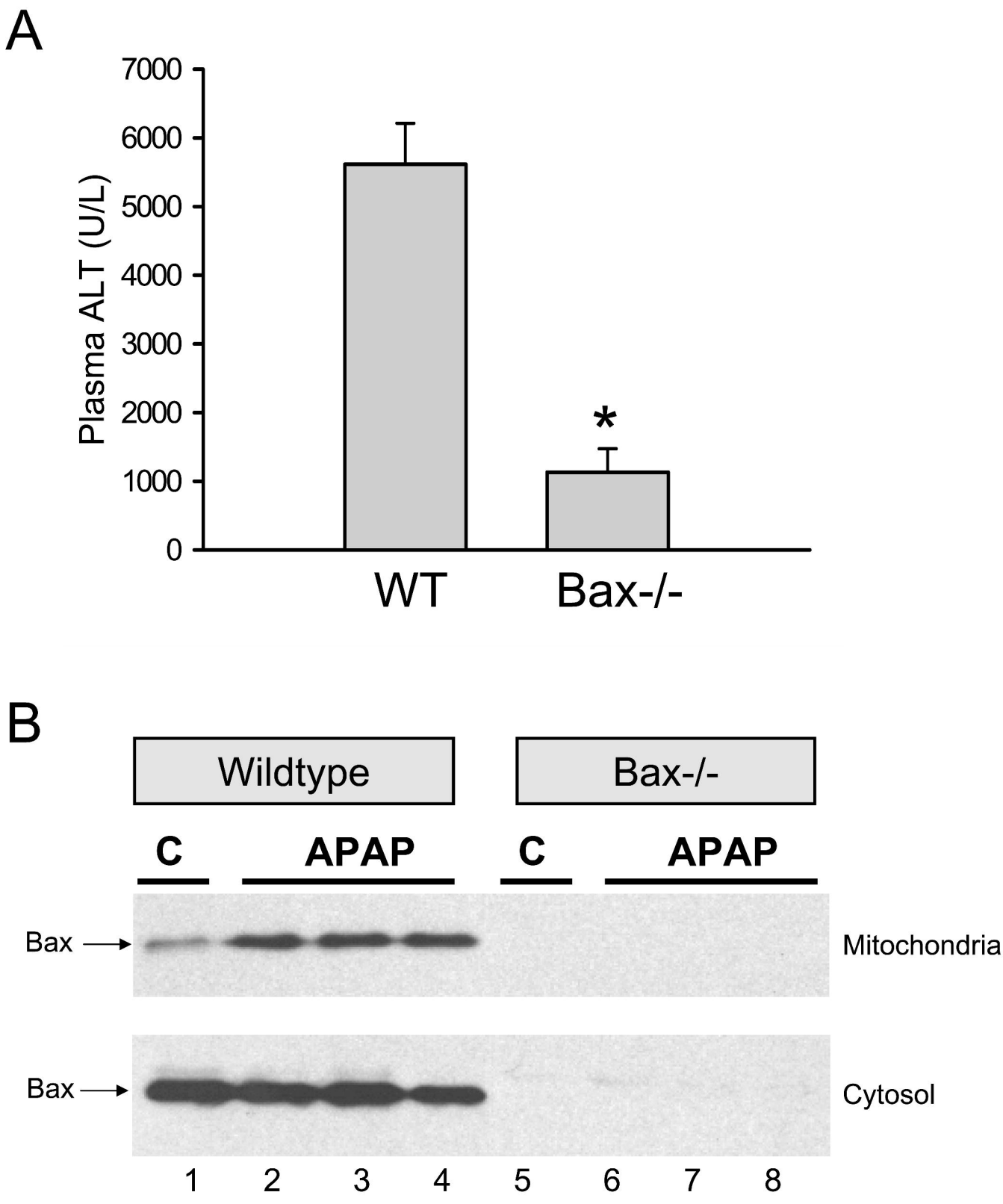
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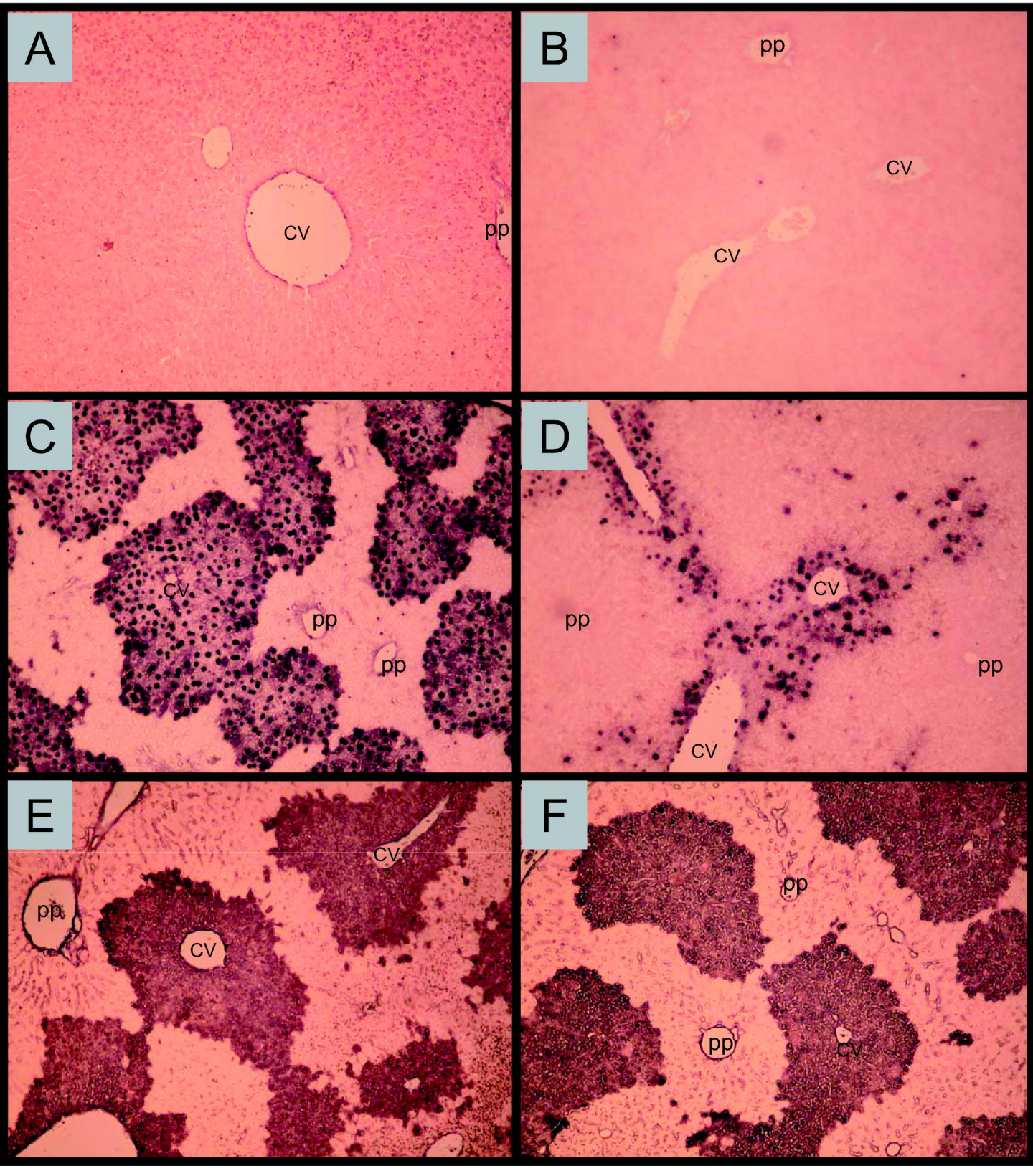
Figure 3:



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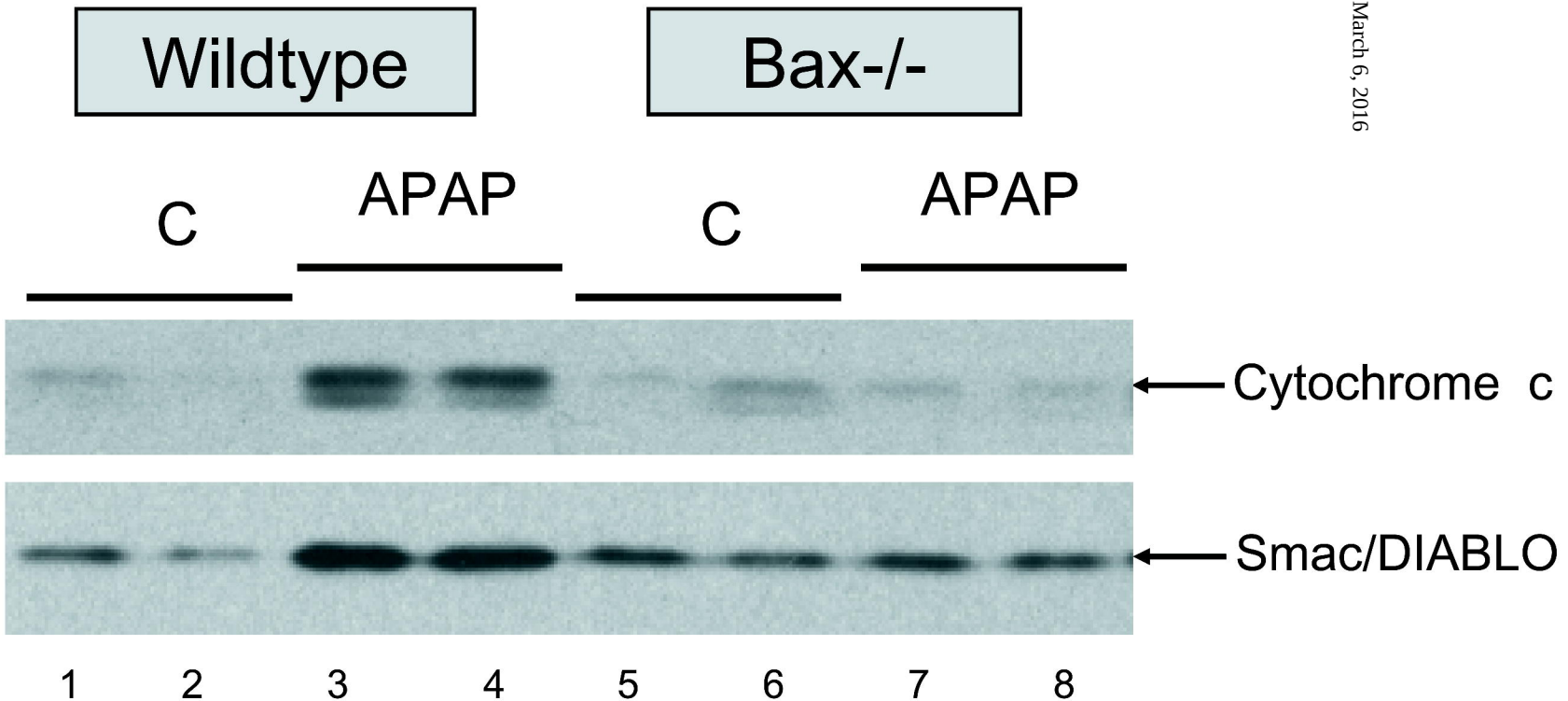

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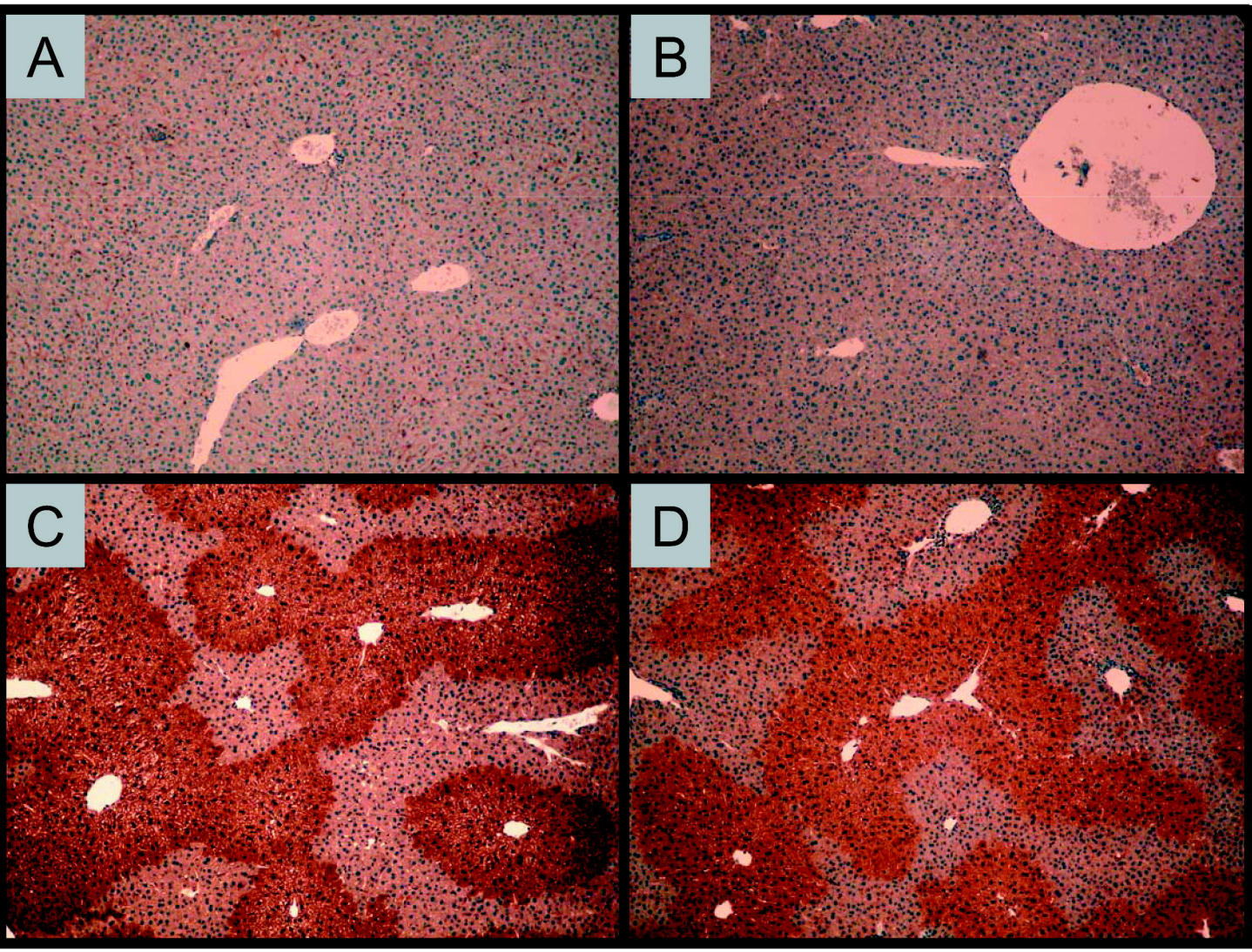


Figure 6

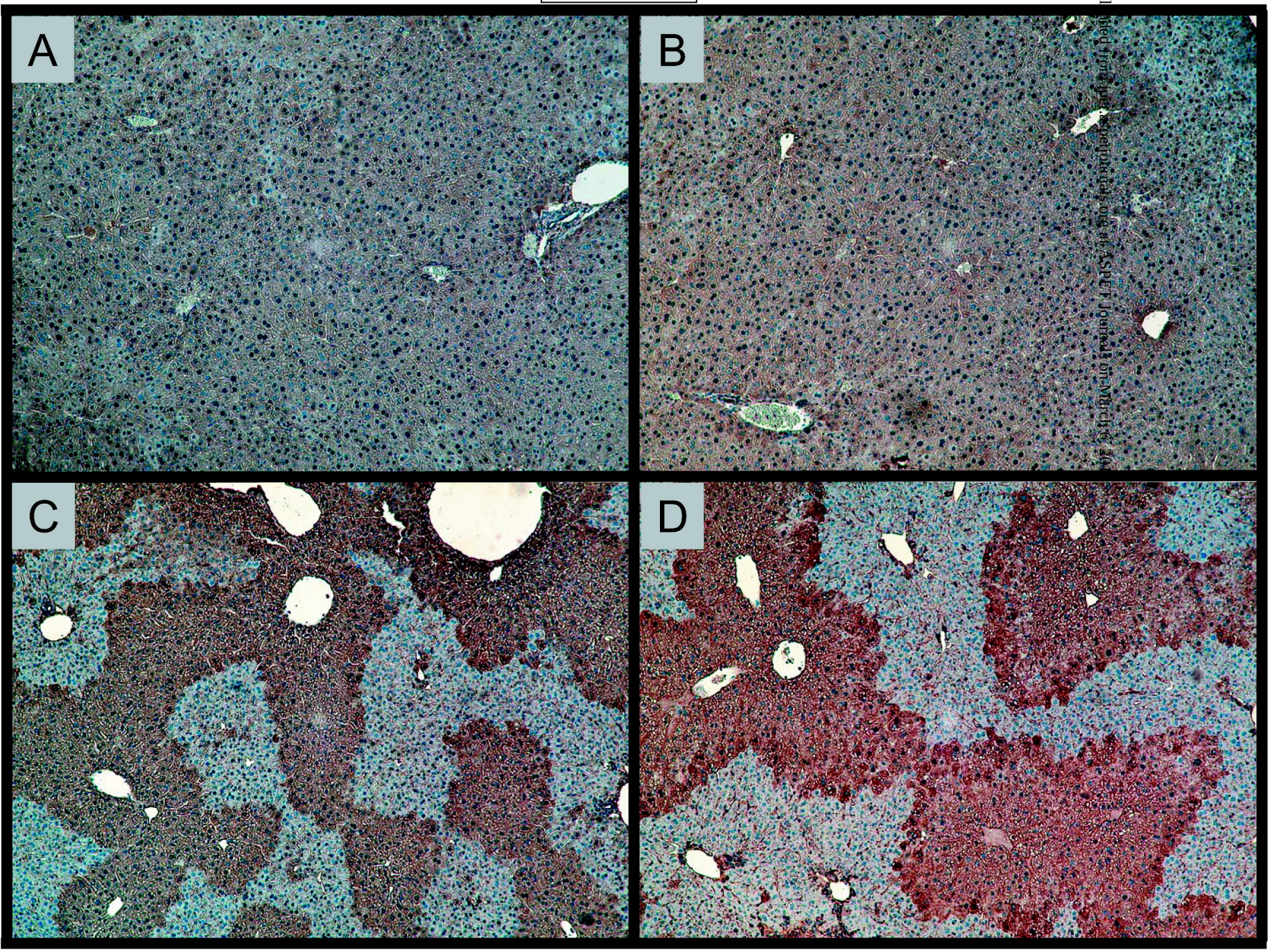
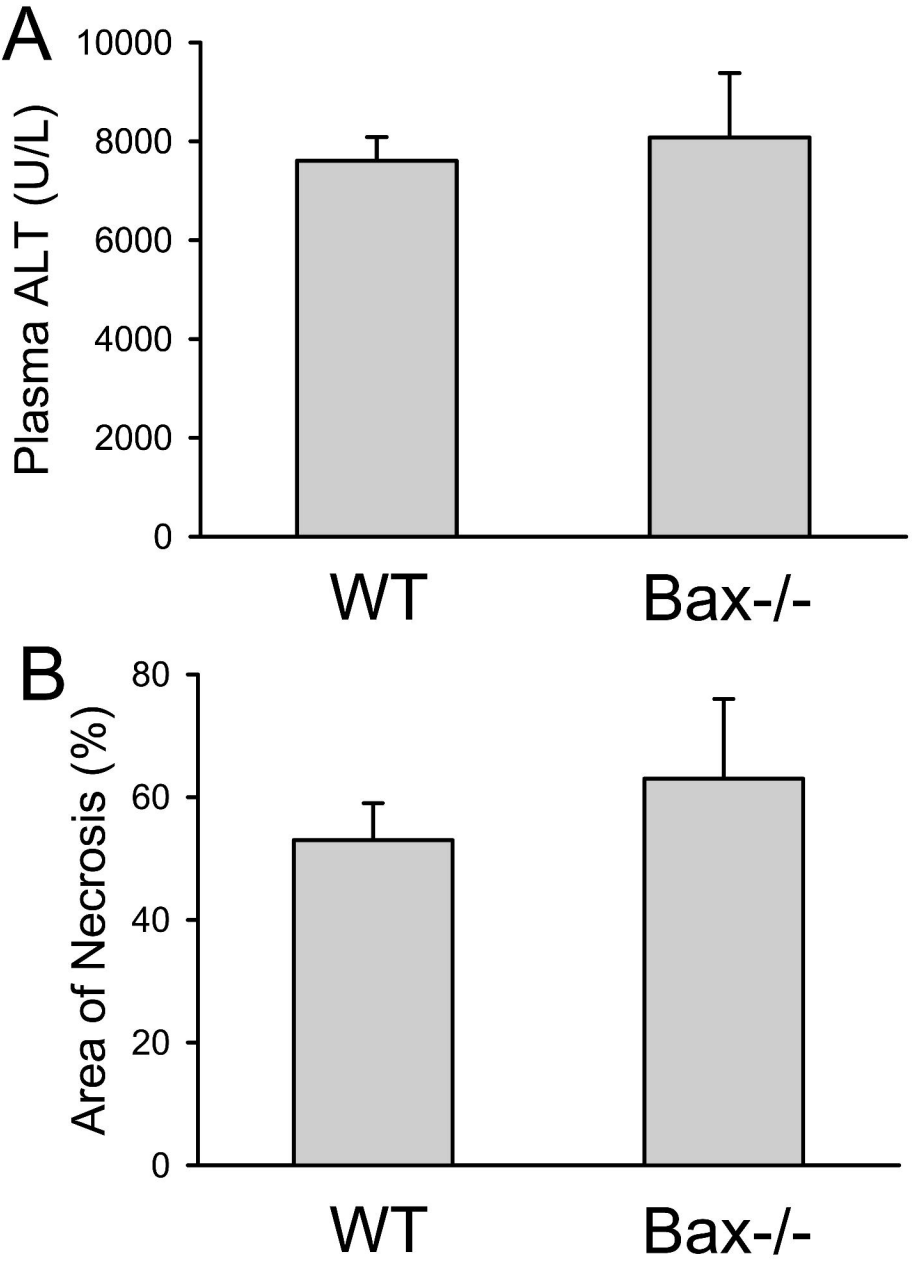


Figure 7: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4000
2000
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WT
Bax-/-
B
Area of Necrosis (%)
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60
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20
0
WT
Bax-/-