Dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways is associated with acetaminophen injury responsive liver regeneration

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Running title page

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ABBREVIATIONS

ALR, augmenter of liver regeneration; ALT, alanine aminotransferase; ARE, antioxidant response element; AST, aspartate aminotransferase; CDK4, cyclin D-dependent kinase 4; ERK1/2, extracellular signal-regulated kinase 1/2; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; JNK, c-Jun N-terminal protein kinase; KEAP1, Kelch-like ECH-associated protein 1; MDM2, mouse double minute 2 homolog; mTOR, mammalian target of rapamycin; NAPQI, N-acetyl-p-benzoquinone imine; NQO1, NAD(P)H quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2-related factor 2; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species.
ABSTRACT

Acetaminophen (APAP) overdose is the leading cause of drug-induced liver injury. Compensatory liver regeneration is crucial for the final outcome of toxicant-induced injury. However, the molecular mechanisms underlying compensatory liver regeneration in mice after APAP-induced liver injury are not completely understood. This study aimed to investigate the role of dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 pathways in APAP injury responsive liver regeneration. We found that mice after 400 mg/kg APAP treatment exhibited massive hepatic toxicity during the first 12 h but responsive liver recovery occurred beyond 24 h as demonstrated by histopathological and biochemical assessments. The expression and nuclear accumulation of NRF2 was increased after APAP treatment. The expression of NQO1, GCLM and HO-1 was inhibited during the first 24 h and then induced to limit oxidative damage. The content of p53 and its downstream target p21 were significantly increased upon APAP exposure and subsequently decreased to normal levels at 48 h. Furthermore, levels of cyclin D1, CDK4, PCNA, and ALR at 48 h were enhanced, suggesting initiation of hepatocyte proliferation and tissue repair. These results demonstrated that dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways was associated with compensatory liver regeneration after APAP-induced acute injury.
Introduction

Acetaminophen (APAP), a widely-used analgesic and antipyretic drug, is relatively safe at recommended doses but an acute or cumulative overdose frequently results in liver injury. Hepatotoxicity induced by APAP is the most common cause of drug-induced acute liver failure in the United States and Great Britain (Larson et al., 2005). APAP toxicity is initiated by accumulation of the toxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is generated by cytochrome P450 enzymes (isoforms CYP2E1, 1A2 and 3A) (Patten et al., 1993). NAPQI subsequently triggers hepatic toxicity through covalently binding with cellular proteins and/or through increasing reactive oxygen species (ROS) leading to apoptosis and hepatocellular necrosis (Saito et al., 2010).

Extensive evidence has demonstrated that compensatory liver regeneration is a critical determinant of the final outcome of toxicant-induced injury, which dictates survival or death (Anand et al., 2003; Mehendale, 2005). Liver regeneration allows for the replacement of necrotic cells and the full recovery of organ function. It is well known that responsive liver regeneration is governed by a complex signal transduction network involving numerous chemokines, cytokines, growth factors, and nuclear receptors (Fausto et al., 1995; Fausto et al., 2006).

The nuclear factor erythroid 2-related factor 2 (NRF2), a master regulator of the antioxidant defense system, mediates a cell survival response. It regulates the expression of a battery of genes encoding intracellular detoxifying enzymes and antioxidant proteins through the antioxidant response element (ARE) (Sykiotis and
Bohmann, 2010). ARE-driven detoxification and antioxidant genes include NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase catalytic and modifier subunits (GCLC and GCLM), and heme oxygenase-1 (HO-1) (Jaiswal, 2004; Kobayashi and Yamamoto, 2005). Most recently, it has been reported that augmenter of liver regeneration (ALR) is also a target gene of NRF2 signaling pathway (Dayoub et al., 2013). Under basal conditions, NRF2 interacts with the actin-anchored protein KEAP1 and is localized in the cytoplasm. In response to oxidative/electrophilic stress, NRF2 is released from KEAP1 and translocates into the nucleus, and subsequently activates ARE-responsive gene expression (Kobayashi and Yamamoto, 2005). Several previous studies have demonstrated that activation of the NRF2 signaling pathway serves to protect animals against liver injury produced by various hepatotoxicants including APAP (Chan et al., 2001; Enomoto et al., 2001). Moreover, NRF2 has been shown to enhance cancer cell proliferation and improve the process of liver regeneration after partial hepatectomy (Beyer et al., 2008; Homma et al., 2009). However, whether KEAP1-NRF2-ARE signaling also participates in liver repair following APAP toxicity has not been determined.

p53 is a tumor suppressor which plays an important role in regulating cell growth, DNA repair, and apoptosis (Vogelstein et al., 2000). p53 is activated in response to a wide variety of stress types, which include but are not limited to DNA damage. Activated p53 can inhibit cell proliferation or trigger cell apoptosis, depending on the severity of DNA damage (Bensaad and Vousden, 2005). p21, one of the main effectors of p53, promotes cell cycle arrest and senescence through
regulation of cyclin-dependent kinases and regulatory proteins. The importance of p21 for the regulation of liver regeneration was confirmed by several previous studies (Lehmann et al., 2012; Buitrago-Molina et al., 2013). Inhibition of p21 leads to hepatocyte proliferation in mice with severe injury allowing animal survival. It has been observed that APAP exposure induced p53 and p21 expression in C6 glioma cells and in mice (Ray et al., 2001; Chiu et al., 2003; Lee et al., 2006). However, the dynamic change of p53/p21 signal during APAP injury responsive liver regeneration in mice remains unknown.

Furthermore, it has been shown that acute liver injury induced by moderate doses of APAP is a dynamic process including initiation of injury, progression of injury, and compensatory liver repair (Chiu et al., 2003; Mehendale, 2005; Bajt et al., 2008). Therefore, we presumed that KEAP1-NRF2-ARE and p53/p21 signaling pathways would be dynamically and coordinately regulated during APAP injury responsive liver regeneration. The aim of this study was to investigate whether the time profile and dynamic regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways was associated with compensatory liver regeneration following APAP-induced liver injury.
Materials and methods

Reagents

Acetaminophen was obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for western blot analysis against total JNK, p-JNK, SIRT1, p-p38, and GAPDH were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-GCLC, anti-p53, and anti-GCLM antibodies were from Abcam (Cambridge, UK). NRF2, NQO1, HO-1, and p21 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other antibodies including KEAP1, p-ERK, ERK, PCNA, cyclin D1, p38, CDK4, and ALR were provided by Shanghai Sangon Biotech Co., ltd. (Shanghai, China). The secondary antibody was obtained from Cell Signaling Technologies.

Animals

Male C57BL/6 mice (6-8 weeks old) were obtained from Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China) and maintained under controlled conditions (22-24°C, 55-60% humidity and 12 h light/dark cycle) with free access to standard food and water. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People’s Republic of China (http://www.most.gov.cn). All animal protocols were approved by the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University.

All animals were fasted overnight before the experiments since fasting allows
lower doses of APAP to be used and results in less variation of the injury (Jaeschke et al., 2011). APAP solution was made fresh in 0.9% saline at 40 mg/ml, and mice were administered at a single dose of 400 mg/kg APAP by intraperitoneal (i.p.) injection. All mice were killed at 0, 6, 12, 24, 48 h after APAP treatment. Serum samples and liver tissues were harvested. A portion of the liver was immediately fixed in 10% buffered formalin for histological analysis and the remaining tissues were flash frozen in liquid nitrogen and stored at -80°C for further use.

**Histological Analysis**

Liver tissues fixed in buffered formalin were embedded in paraffin, cut into 3 µm thick sections, and stained with hematoxylin and eosin (H&E). HE-stained liver sections were used to assess liver damage using a LEICA DM5000B microscope (Leica, Heidelberg, Germany). Three tissues collected at various time points were examined and found to exhibit similar injury for each treatment group at the same time point. Thus, one representative image was presented.

**Biochemical analysis**

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured to indicate APAP-induced liver injury and/or liver recovery. ALT and AST activities of serum obtained at 0, 6, 12, 24, 48 h after APAP treatment were determined using a Beckman Synchron CX5 Clinical System and a commercial reagent kit (Kefang biotech, Guangzhou, China) according to the manufacturer’s
protocol. To assess oxidative stress induced by APAP, levels of reduced glutathione (GSH), hydrogen peroxide (H₂O₂), and malondialdehyde (MDA) were also determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Western Blot Analysis**

Western blot analysis was performed as described in our previous report (Chen et al., 2014). Briefly, RIPA protein extracts were prepared from frozen liver tissues, and protein concentration was determined using the bicinchoninic acid method (Thermo Scientific, Rockford, IL). Equivalent amounts of protein extracts were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, USA). Phosphorylated proteins were blocked with 5% BSA in Tris-buffered saline, and other proteins with 5% non-fat milk in Tris-buffered saline. Membranes were incubated overnight at 4°C with primary antibodies, followed by secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Protein-antibody complexes were detected using an electrochemiluminescence (ECL) kit (Engreen Biosystem, Beijing, China) and exposed to an X-ray film (GE Healthcare, Piscataway, NJ, USA). The intensity of protein bands was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

**Statistical Analysis**
Data are presented as the mean ± SEM. Statistical analysis was performed by one
way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test
using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). \( P \) value less
than 0.05 was considered statistically significant.
Results

APAP-induced liver injury is followed by compensatory liver regeneration in mice.

In this study, 400 mg/kg APAP treatment resulted in massive hepatic toxicity as revealed by H&E staining of liver sections (Figure 1A) and increased serum ALT and AST activities (Figure 1B). Centrilobular necrosis was clearly evident at 6 h after APAP treatment, peaked between 12 and 24 h. Compared to the first 24 h, much less hepatocellular injury and necrosis was observed at 48 h, but was not yet completely resolved. To assess liver injury after APAP treatment, time courses of serum ALT and AST activities were measured over 48 h. The ALT and AST levels were increased in parallel to the area of hepatic necrosis. The ALT and AST activities were significantly elevated with a peak at 12 h after APAP dosing, subsequently reduced at later time points, and then tended toward baseline levels by 48 h as previously described (Bajt et al., 2008). These observations indicated that APAP-induced acute liver injury mainly occurred during the first 12 h following APAP challenge. Furthermore, APAP treatment caused a significant decrease in liver and mitochondrial reduced GSH levels in livers at 6 h and 12 h after APAP treatment, suggesting an excess NAPQI generation leading to GSH depletion (Figure 1C). However, levels of reduced GSH were increased beyond 12 h and restored at 48 h. Levels of mitochondrial H$_2$O$_2$ and total liver MDA were rapidly elevated after APAP challenge (Figure 1D-1E). Following the maximum levels at 6 h, H$_2$O$_2$ and MDA subsequently reduced and returned to normal levels by 48 h, which reflected
decreased oxidative stress. On the other hand, JNK activation is known to be a critical step in the intracellular signaling related to APAP-induced liver injury, along with activation of ERK1/2 (Gunawan et al., 2006; Saito et al., 2010). As expected, western blot analysis of total and phosphorylated JNK indicated rapid activation of JNK in APAP-treated mice within 6 h after APAP challenge (Figure 2). However, level of JNK phosphorylation was drastically decreased in a time-dependent manner at later time points, and was low or undetectable at 48 h. Elevated p-JNK levels were consistent with increased p-c-jun levels, suggesting that p-JNK was correlated with enhanced activity. ERK1/2 was also significantly activated with an early peak of activation at 6 h when mice were exposed to APAP. Interestingly, ERK2 (p42) appeared to be preferentially activated over ERK1 (p44) upon APAP exposure. Similar to p-JNK, p-ERK1/2 levels declined to basal levels by 48 h. Additionally, p38 MAPK was phosphorylated and active in normal liver, but rapidly inactivated between 6 and 24 h after APAP treatment and re-activated at 48 h.

Dynamic regulation of KEAP1-NRF2-ARE pathway is related to APAP injury responsive liver regeneration.

The expression levels of proteins involved in KEAP1-NRF2-ARE pathway were measured to explore whether KEAP1-NRF2-ARE signaling pathway participated in compensatory liver regeneration following acute APAP-induced liver injury. The results showed that level of KEAP1 protein was down-regulated between 6 and 24 h after APAP treatment, but up-regulated at 48 h (Figure 3). Treatment with 400
mg/kg APAP in mice significantly induced NRF2 expression with a peak at 12 h and facilitated nuclear accumulation of NRF2 with a peak at 6 h as previously described (Goldring et al., 2004; Copple et al., 2008). The protein expression of NRF2 target genes including Ho-1, Nqo1, Gelm, Gclc, and Alr in APAP-treated mice was also examined using western blotting. Compared to basal levels observed in uninjured livers, APAP exposure firstly reduced HO-1 protein levels between 6 and 24 h, followed by a marked induction at 48 h. GCLM levels remained unchanged during the first 12 h after APAP challenge, and then were significantly decreased at 24 h. The expression of NQO1 was reduced at 6 h and 12 h. Both NQO1 and GCLM levels were up-regulated at 48 h after APAP treatment. Additionally, a biphasic regulation of ALR by APAP was also observed including inhibition phase from 6 to 24 h and induction phase between 24 and 48 h during APAP injury responsive liver regeneration. However, the protein expression of GCLC was presented a single-phase up-regulation over a time course of 0 to 48 h following APAP challenge. The present data implicated that NRF2 target genes were suppressed at the early stage of APAP-induced liver injury but up-regulated at later stage.

Dynamic regulation of p53/p21 signaling pathway is associated with compensatory liver regeneration following APAP-induced liver injury.

To explore whether p53/p21 signaling was associated with liver repair after APAP-induced liver injury in mice, p53 and p21 expression was analyzed. p53 was normally maintained at low levels in uninjured livers. As expected, APAP treatment
induced p53 expression with the peak p53 at 6 h and 12 h (**Figure 4**). Beyond 12 h, p53 level was markedly reduced. Similar to p53, p21 showed a significant induction at 6 h after APAP exposure followed by a time-dependent decline at later time points, and returned to normal levels at 48 h. Treatment with APAP resulted in an inhibition of Sirtuin 1 (SIRT1) expression in a time-dependent manner. Furthermore, the expression of cyclin D1, CDK4, and PCNA in APAP-injured livers was measured. Treatment with APAP resulted in a decrease in cyclin D1 and PCNA levels. However, there was a further significant decrease of cyclin D1 protein expression at 24 h followed by an increase to baseline levels by 48 h. PCNA level was also increased at 48 h compared to other time points at early stage, but not completely restored to normal levels observed in uninjured livers. The protein expression of CDK4 was unchanged between 0 and 24 h after APAP overdose, but significantly up-regulated at 48 h. Detailed analysis of cell cycle protein levels over a time course from 0 to 48 h revealed that the mice experienced transient impairment of hepatocyte proliferation at the early stage of APAP toxicity, and this inhibition was overcome by 48 h leading to compensatory liver regeneration.
Discussion

APAP overdose is the leading cause of drug-induced liver injury and a significant public health concern. Previous studies have revealed that compensatory tissue repair following toxicant challenge plays a crucial role in impacting the final outcome of liver injury, either recovery from injury or injury progression leading to liver failure (Mehendale, 2005; Bajt et al., 2008). In the current study, the role of dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 pathways in APAP injury responsive liver regeneration was evaluated.

The results showed that area of hepatic necrosis and activities of ALT and AST were significantly increased during the first 12 h suggesting initiation and progression of liver injury, and subsequently reduced at later time points reflecting regression of injury. In addition, a decline of oxidative stress was observed as demonstrated by increased hepatic and mitochondrial GSH levels, decreased mitochondrial H$_2$O$_2$, liver MDA, p-JNK, p-c-jun, and p-ERK1/2 levels beyond 12 h. p38 MAPK, involved in the liver regeneration networks, was rapidly inactivated after APAP overdose and then re-activated at 48 h, which was supported by previous observations related to the regenerating liver after partial hepatectomy (Liao et al., 2004). These results suggest that compensatory liver regeneration occurs at later stage of liver injury induced by APAP (Figure 5).

Further results clearly demonstrated that treatment with 400 mg/kg of APAP resulted in KEAP1 inhibition and NRF2 up-regulation between 6 and 12 h. Despite the marked NRF2 nuclear translocation occurring after APAP challenge, none of four
NRF2 target genes was significantly induced during this period. In contrast, HO-1, NQO1, and GCLM levels were down-regulated during the first 24 h. Translocation of NRF2 was uncorrelated with unchanged or/and decreased expression of these NRF2 downstream target genes, suggesting that there were some delays between NRF2 nuclear translocation and its target gene induction and protein translation. However, during the later stage of liver injury, the protein expression of detoxification and antioxidant genes Ho-1, Nqo1, Gclc, and Gclm was significantly induced to promote liver repair. Glutamate cysteine ligase, composed of a catalytic (GCLC) and a modifier (GCLM) subunit, catalyzes the rate-limiting step in GSH biosynthesis. Thus increased GSH levels at later stage were correlated with significantly up-regulated GCLC and GCLM expression. Our data indicated that two stages including induction phase and repression phase in expression of KEAP1 and NRF2 target genes were present during APAP-induced liver injury in mice. The KEAP1-NRF2-ARE signaling pathway may regulate an adaptive response to protect against oxidative damage following APAP challenge. In addition, ALR was identified to promote liver regeneration after partial hepatectomy (Polimeno et al., 2011), and to augment hepatocyte proliferation when the regenerative process was primed (Francavilla et al., 1994). ALR may exhibit a beneficial effect via NRF2 pathway on compensatory liver regeneration following APAP-induced liver injury.

On the other hand, the expression of p53 and p21 was significantly induced after APAP challenge and subsequently down-regulated to normal levels at 48 h. SIRT1 was reported to regulate acetylation of p53 on Lys382 residue and p53-dependent
apoptosis (Cheng et al., 2003). Very recently, SIRT1 has been identified to regulate regenerative response in the liver through FXR and mTOR signaling pathway (García-Rodríguez et al., 2014). Whether SIRT1 expression is affected by APAP is not yet reported. In the present study, decrease in SIRT1 levels followed by elevated p53 and p21 levels was observed upon APAP exposure. Additionally, JNK phosphorylation induced by APAP can activate and stabilize p53 leading to reduced ubiquitination and proteasomal degradation as previously described (Fuchs et al., 1998). Our observations showed a substantial over-expression of p53 and p21 during the first 24 h, which may be the reason for the inhibition of cell cycle activation as indicated by decreased cyclin D1 and PCNA expression. Levels of p53 and p21 were declined between 24 and 48 h, but cyclin D1, CDK4, and PCNA expression was rapidly increased during this period, which allowed regeneration to proceed in APAP-treated mice. Previous study showed that the expression of cyclin D1 mRNA and PCNA protein showed a significant increase at 24 h following 200 mg/kg APAP treatment (Bajt et al., 2008), which is different from our observations. This can be explained by difference in APAP doses since tissue repair is a toxicant dose-dependent dynamic process (Mehendale, 2005). With each increment in APAP dose, a corresponding delay in the onset of tissue repair would be observed. APAP overdose resulted in a strong and robust induction of p53/p21 in mice with acute liver injury during the early time points, which would subsequently impair liver regeneration. When injury regression occurred at later time points, levels of p53 and p21 were returned to baseline. Together, these data indicate that the degree of liver
injury is related to the strength of p53/p21 induction in the liver and its effect on hepatocyte proliferation.

Recently, a correlation between NRF2 and p53 in response to oxidative stress has been reported (Faraonio et al., 2006; You et al., 2011; Chen et al., 2012). In this study, at the early stage of liver injury caused by APAP, high levels of ROS may induce p53 expression and activation, which would subsequently inhibit a battery of cytoprotective genes mediated by NRF2, such as Ho-1, Nqo1, Gclm, and Alr, and promote cell cycle arrest and apoptosis. Under low levels of ROS at later stage, normal levels of p53/p21 could induce expression of antioxidant genes such as NRF2 target genes to protect against ROS-mediated damage and restore cell cycle progression. Collectively, our results suggest that dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways is associated with compensatory liver regeneration following APAP-induced liver injury in mice.
Authorship Contributions

Participated in research design: Bi, Huang, Fan.

Conducted experiments: Fan, Chen, Tan, Zeng, Jiang, Y. Wang.

Contributed to new reagents and analytic tools: Y. T. Wang, Hou.

Performed data analysis: Fan, Bi.

Wrote or contributed to the writing of the manuscript: Fan, Bi.
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Footnotes

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Figure legends

**Figure 1.** APAP-induced liver injury was followed by compensatory liver regeneration in mice. (A) H&E-stained liver sections, (B) serum ALT and AST activities, (C) total liver and mitochondrial reduced GSH levels, (D) mitochondrial H$_2$O$_2$ levels, (E) total MDA levels from APAP-treated mice over a time course from 0 to 48 h after 400 mg/kg APAP challenge. Data are expressed as the mean ± SEM (n=5). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus 0 h, #*$P<0.05$, ##*$P<0.01$, ###*$P<0.001$ versus 48 h.

**Figure 2.** JNK and ERK were activated, but p38 MAPK was inactivated following APAP challenge. (A) Western blot analysis of p-JNK, JNK, p-c-jun, p-ERK, ERK, p-p38 and p38 protein expression using RIPA extract of APAP-treated livers obtained at various time points after APAP challenge. (B) Densitometric analysis of western blots. Data are expressed as the mean ± SEM (n=3). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus 0 h, #*$P<0.05$, ##*$P<0.01$, ###*$P<0.001$ versus 48 h.

**Figure 3.** Dynamic regulation of KEAP1-NRF2-ARE signaling pathway was involved in compensatory liver regeneration following APAP-induced liver injury in mice. (A) Western blot analysis of KEAP1, NRF2, GCLC, GCLM, NQO1, HO-1, ALR, and GAPDH protein expression in APAP-treated mice at various time points after APAP challenge. (B) Densitometric analysis of western blots. Data are
expressed as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 versus 0 h, 
#P<0.05, ##P<0.01, ###P<0.001 versus 48 h.

Figure 4. Dynamic regulation of p53/p21 signal participated in hepatocyte proliferation following APAP-induced liver injury in mice. (A) Western blot analysis of p53, p21, SIRT1, cyclin D1, CDK4, PCNA, and GAPDH protein expression in APAP-treated mice over a time course from 0 to 48 h after APAP challenge. (B) Densitometric analysis of western blots. Data are expressed as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 versus 0 h, #P<0.05, ##P<0.01, ###P<0.001 versus 48 h.

Figure 5. Schematic presentation of proposed two-stage model for APAP-induced hepatotoxicity. At stage I, liver injury induced by APAP overdose was initiated by NAPQI and further progressed upon exposure to high oxidative stress. During the first 24 h, ROS was elevated as indicated by decreased reduced GSH, increased H₂O₂ and MDA levels, and a prompt activation of JNK and ERK. KEAP1-NRF2-ARE pathway was inhibited by APAP, and p53/p21 signaling was up-regulated followed by decreased expression of cell cycle proteins. During stage II, compensatory liver regeneration response was stimulated by infliction of liver injury, which resulted in rapid regression of injury to allow animal recovery from injury and survival. Between 24 and 48 h, mitochondrial H₂O₂, total MDA, p-JNK and p-ERK levels markedly reduced followed by elevated reduced GSH, reflecting a decline in
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oxidative stress level. In the recovery stage, KEAP1-NRF2-ARE pathway was induced, and p53/p21 signaling was down-regulated accompanied by increased expression of cell cycle proteins.
Figure 2

A

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B

- Relative protein expression graph showing changes over time (0, 6, 12, 24, 48 hours) for different proteins:
  - p-JNK1/JNK1
  - p-JNK2/JNK2
  - p-c-jun
  - p-ERK1/ERK
  - p-ERK2/ERK
  - p-p38/p38

- Significant changes are indicated by asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), ### (p < 0.0001).
Figure 3

A

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</table>

B

Relative protein expression over time (h)

- KEAP1
- total NRF2
- nuclear NRF2
- NQO1
- HO-1
- GCLC
- GCLM
- ALR

Significance levels:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- **** p < 0.0001

Note: The image contains a graph showing the relative protein expression levels of various proteins over time (0 to 48 hours) after APAP treatment.
Figure 4

A

APAP treatment (h)

0  6  12  24  48

p53

p21

SIRT1

Cyclin D1

CDK4

PCNA

GAPDH

B

Relative protein expression

Time (h)

0  6  12  24  48

p53

p21

SIRT1

CDK4

Cyclin D1

PCNA

* * *
Figure 5

**Stage I**
Initiation and progression of liver injury

- ↑ ROS (↓ GSH, ↑ H₂O₂, ↑ MDA)
- ↑ p-JNK, p-ERK
- ↓ KEAP1-NRF2-ARE
- ↑ p53/p21
- Cell proliferation associated proteins (Cyclin D1, CDK4, PCNA, ALR)

**Stage II**
Compensatory liver regeneration and regression of liver injury

- ↓ ROS (↑ GSH, ↓ H₂O₂, ↓ MDA)
- ↓ p-JNK, p-ERK
- ↓ KEAP1-NRF2-ARE
- ↓ p53/p21
- Cell proliferation associated proteins (Cyclin D1, CDK4, PCNA, ALR)

**APAP-induced acute liver injury**

- Necrosis or apoptosis
- Recovery from injury and survival