

Chlorogenic Acid Alleviated Liver Dysfunction Through Activating PINK1/Parkin Dependent Mitophagy

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Abstract

Background: Chlorogenic acid (CGA) is a natural polyphenolic compound with anti-inflammatory, antioxidant effects. It could improve mitochondrial dysfunction that was a key feature of acetaminophen (APAP) -induced liver injury. This study aimed to identify whether promoting mitophagy was associated with the hepatocyte protection for CGA.

Methods: Acute hepatic injury model was induced by APAP in mice after CGA administration for 14 days. Survival rate was recorded within 24h of modeling. Serum aminotransferase, hepatic histopathology and TUNEL assays were simultaneously performed. The expression of apoptosis-related proteins (Bax and Bcl-2) and mitophagy-related genes and proteins (LC3, P62, PINK1 and Parkin) were analyzed. The fluorescence co-localization of LC3 and Tom20 was analyzed with immunofluorescence.

Results: Compared with APAP group, CGA pretreatment significantly increased survival rate of APAP-induced mice, inhibited the activity of ALT, AST and LDH in serum, and alleviated pathological features of liver such as inflammatory cell infiltration, necrosis of liver cells and vacuolation ($p < 0.05$). Moreover, our data from the TUNEL and western blotting analysis showed that CGA significantly decreased the number of apoptotic cells and reversed the elevated Bax level and decreased Bcl-2 level ($p < 0.05$). Furthermore, we found that CGA promoted the fluorescence co-localization of LC3 and Tom20 and enhanced the protein expression of LC3 ($p < 0.05$). Finally, CGA significantly promoted mitophagy by exhibiting the increased gene and protein expression of PINK1 and Parkin.

Conclusions: Our results demonstrated that CGA promoted PINK1/Parkin dependent mitophagy and inhibited hepatic apoptosis to exert protection against liver damage in APAP-induced mice.

1. Introduction

Acetaminophen (APAP) is the most commonly used antipyretic and analgesic. APAP could cause severe liver, kidney and heart injury or organ failure at a single overdose exceeding 15 to 25 g, although it is generally safe used at therapeutic dose (<4 g every 24 h) [1–3]. Acute liver injury is the most common side effect of acetaminophen overdose because liver is one of the most sensitive organ to acetaminophen. Recent studies have suggested multiple molecular mechanisms that contribute to APAP hepatotoxicity, including oxidative stress, sterile inflammation, mitochondrial dysfunction, especially impaired mitophagy [4] and apoptosis [5]. It is well known that APAP induced liver toxicity starts from the production of toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). Excessive NAPQI accumulation leads to glutathione (GSH) in the liver and leads to mitochondrial dysfunction and subsequent oxidative stress [6]. Experiments have shown that autophagy can selectively remove NAPQI and reduce liver damage. On the contrary, autophagy inhibitor Chloroquine can aggravate APAP-induced liver damages [7, 8].

Mitochondria are highly dynamic organelles that govern redox homeostasis, innate immunity and apoptotic cell death [9]. In liver cells, mitochondria are the starting point for inflammatory signals and

oxidative stress responses [10]. Mitochondrial reactive oxygen species (ROS) is one of the major sources of cellular ROS, which can damage lipids, proteins and DNA, leading to mitochondrial dysfunction [11, 12]. It is now generally accepted that autophagy can clear the affected damaged mitochondria, reduce the production of ROS and eliminate inflammasomes (such as NLRP3 inflammasomes) [13]. At present, a large number of studies have shown that APAP can induce hepatocyte apoptosis [14–17]. When mitophagy is damaged, the impaired Mitochondria cannot be removed and accumulate in large quantities, which lead to a large number of hepatocytes apoptosis to aggravate the degree of liver injury.

Autophagy is a lysosomal degradation process responsible for degrading dysfunctional proteins and organelles [18]. Mitophagy, a selective autophagy of mitochondria, plays an important protective role in preventing DILI by timely removing damaged mitochondria [19]. Generally, mitophagy involves mitochondrial fission, autophagosome formation, and fusion with lysosome in turn [20]. These processes are regulated by phosphatase and tensin homolog (PTEN)-induced kinase 1 (Pink1)/Parkin pathway. Briefly, Pink1 locates on the outer mitochondrial membrane of damaged mitochondria from the inner membrane, then removes the damaged mitochondria by recruiting Parkin in response to various stimuli [21]. Importantly, suppression of Pink1/Parkin augments acetaminophen hepatotoxicity via impairment of hepatic mitophagy, which indicates that Pink1/Parkin-mediated mitophagy plays a key role in alleviating acetaminophen hepatotoxicity [22, 23]. Therefore, it is critically important to prevent acetaminophen hepatotoxicity through precise regulation of Pink1/Parkin pathway. Intriguingly, a recent study found that knockdown of Parkin augmented endplate chondrocyte apoptosis in H₂O₂-induced mitochondrial dysfunction cell model, which implied that Parkin-mediated mitophagy might involve in regulating apoptosis [24]. However, targeting mitophagy-mediated apoptosis for relieving DILI remains largely undetermined.

Chlorogenic acid (CGA, C₁₆H₁₈O₉) is a dietary polyphenol derived from coffee, apple, blueberry, tea and some natural medicines with multiple biological activities. Growing evidence has proved the protective effect of CGA on acetaminophen hepatotoxicity which involves multiple mechanisms including anti-oxidation, anti-inflammatory and anti-apoptosis [25–28]. More specifically, they are concerned with attenuating liver mitochondrial injury and reducing mitochondrial HSP60 release, activating Nrf2 antioxidative signaling pathway, inhibiting MAPK signaling pathway, inhibiting CYP2E1 and CYP1A2 enzymatic properties and suppressing TLR3/4 and NF kappa B signaling pathway [25–31]. It's well known that mitophagy defect can perturb mitochondrial function and cause progressive accumulation of reactive oxide species (ROS) and the inability to supply energy, leading to oxidative stress, sterile inflammation and cell apoptosis, reinforcing a vicious cycle. However, whether promoting mitophagy was associated with the hepatocyte protection for CGA remains unclear. In this study, we determined the role of CGA on preventing acetaminophen hepatotoxicity based on targeting mitophagy-mediated apoptosis in mice. Our data showed pretreatment with CGA provided a protective role in APAP-induced acute hepatotoxicity mice via activating Pink1/Parkin-dependend mitophagy and inhibiting hepatic apoptosis.

2. Materials And Methods

2.1 Drugs and reagents

Chlorogenic acid (CGA, purify \geq 98%) was purchased from Baoji Herbest Bio-Tech Co., Ltd. (Baoji, China). Acetaminophen (APAP) was obtained from Shanghai Johnson Pharmaceutical Co., Ltd. (Shanghai, China). Ammonium glycyrrhizinate (AG, purify \geq 98%) was provided by ALFA Biological Technology Co., Ltd. (Chengdu, China). Commercial assay kits for alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) were supplied by Medical System Biotechnology Co., Ltd. (Ningbo, China). Cell Total RNA Isolation Kit was acquired from Chengdu Foregene Biotechnology Co., Ltd. (Cat. RE-03113). Anti-Tom20 was provided by Santa Cruz Biotechnology, Inc. Anti-SQSTM1/p62 and anti-Parkin were offered by Cell Signaling Technology (Danvers, MA, USA). Anti-LC3, anti-Bcl2 and anti-Bax were furnished by Proteintech Biological Technology Co., Ltd. (Wuhan, China). Anti-PINK1 was purchased from Abcam (Cambridge, UK). Anti-glyceraldehyde phosphate dehydrogenase (GAPDH) was produced by Servicebio (Wuhan, China). The BCA protein assay kit was acquired from Multi Sciences Biotech CO., Ltd. (Hangzhou China).

2.2 Animals

100 male 4-6 weeks Kunming mice (20 ± 2 g) were obtained from Byrness Weil biotech Co., Ltd. (Chengdu, China). All mice were acclimated for 3 days in a constant temperature and humidity room ($24^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $50\% \pm 10\%$ humidity) with standard diet, water and a 12-h light/dark cycle (lights on at 8:00 am and lights off at 8:00 pm).

2.3 APAP-induced acute hepatotoxicity mice model and treatment

All mice were randomly divided into five groups ($n=20$), the control (Ctrl) group, the APAP group (300 mg/kg), the APAP (300 mg/kg) +AG group (200 mg/kg), and the APAP (300 mg/kg) + CGA group (20 mg/kg or 40 mg/kg). All the material under study is endotoxin free. Mice in the intervention group were respectively pre-administered with CGA (20 mg/kg or 40 mg/kg) and AG (200 mg/kg) for 14 consecutive days. Simultaneously, the control and APAP groups were congruously administrated with same volume 0.9% saline. On day 15, mice were administered a single dose of APAP (300 mg/kg) to induce APAP acute hepatotoxicity except the control group was administered 0.9% saline[32]. Then, all mice were sacrificed on day 16 and serum and liver tissues were collected.

2.4 Mortality in mice assay

After APAP (300mg/kg) treatment, the death period of mice was recorded within 24 h.

2.5 Biochemical analysis for blood

Serum was collected after centrifugation at 3500 rpm for 10 min at room temperature. Serum enzymatic activities of ALT, AST and LDH were measured with a HITACHI 7180 automatic biochemistry analyzer (Hitachi, Japan).

2.6 Histopathology of liver tissue and TUNEL assay

Fresh liver tissues were immediately fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and then cut into 4- μ m-thick sections (n=5). The tissues were stained with hematoxylin and eosin (HE) for histological examination under a light microscopy. The histological scores (including inflammation, necrosis and apoptosis) indicating the degree of liver injury were determined according to Suzike's standard [33]. Simultaneously, these slides were subjected to terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) for analyzing hepatocytes apoptosis according to the manufacturer's instructions of a TUNEL apoptotic detection kit. Images were captured by a fluorescence microscopy (Leica Microsystems Wetzlar German), and calculated semi-quantitatively at magnification $\times 200$ to count the positive cells.

2.7 qRT-PCR analysis

Total RNA was extracted from liver samples using Trizol reagent. cDNA was synthesized with the SuperScript® IV First-Strand Synthesis System (Bio-Rad, Singapore). qRT-PCR was performed in the SYBR-Green PCR kit on Applied Biosystems StepOnePlus system. β -actin was used as the invariant control. The target gene expression levels by the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used in this study are as follows: PINK1: Forward 5'-AGACTCCAGTTCTCGCCT-3', Reverse 3'-AGGGACAGCCATCTGAGTCC-5'; Parkin: Forward 5'-AGCCAGAGGTCCAGCAGTT-3', Reverse 3'-CTGGCACTCACCCTCATCC-5'; p62: Forward 5'-AGATAGCCTTGGAGTCGGTG-3', Reverse 3'-CCGGGGATCAGCCTC TGTAG-5'; LC3- β : Forward 5'-ACCCTAACCCATA GGAGCC-3', Reverse 3'-TGCAAGCG CCGTCTGATTA-5'; β -actin: Forward 5'-GCTCCGGCATGTGCAAAG-3', Reverse 3'-TTCC CACCATCACACCCTGG-5'.

2.8 Protein preparation and western blotting analysis

The total proteins of liver were extracted by RIPA lysis buffer containing protease and phosphatase inhibitor (100:1:1). Protein concentrations were determined using the BCA protein assay kit. Equal amounts of total protein (30 μ g) were separated by 8%-15% SDS-PAGE gels before transferred to the PVDF membranes. Then, the membranes were blocked with 5% fat-free milk at room temperature for 90 min and incubated with primary antibody at 4°C overnight, including anti-GAPDH (1:5000), anti-Parkin (1:1000), anti-PINK1 (1:1000), anti-p62 (1:1000), anti-LC3 (1:1000), anti-Bcl2 (1:1000) and anti-Bax (1:1000). After washing with TBST, the membranes were incubated with secondary goat anti-rabbit IgG (1:5000) or anti-mouse IgG (1:5000) at room temperature for 90 min, and were conjugated with horseradish peroxidase. Then, proteins were detected by chemiluminescence reagent. The relative protein levels were normalized to GAPDH level.

2.9 Immunohistochemistry analysis

The liver tissue was cut into 4- μ m-thick sections then deparaffinization with xylene and gradient ethanol, antigen retrieval with trisodium citrate dihydrate for 30 minutes and blocked with 1% BSA at room

temperature for 30 min. the sections were incubated with primary anti-Tom20 (1:100) and anti-LC3 β (1:300) at 4°C overnight and then with secondary antibodies (1:500) in the dark for 1 h. Hoechst 33258 was used to counterstain the nucleus. At last, the sections were mounted with anti-fluorescence quenching sealer and observed by confocal microscopy (Olympus, Japan).

Statistics

Statistical analysis was performed by one-way analysis of variance (ANOVA) and all values were expressed as mean \pm standard error of the mean (SEM), with the exception of survival rate. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Chlorogenic acid alleviated APAP-induced acute hepatotoxicity in mice

To confirm the potential hepatic protective effects of CGA, mice were pretreated with CGA (20 or 40 mg/kg) and AG (200 mg/kg) for 14 days prior to APAP administration (300 mg/kg). Firstly, we evaluated protective effect of CGA on the survival rate. As shown in Fig. 1A, APAP administration resulted in an obvious decline in survival rates to 65% within 6 h, which remained at this level up to 24 h. Pretreatment of CGA (40 mg/kg) significantly increased survival rates compared with APAP alone ($p < 0.05$). Second, the effects of CGA on the degree of liver function injury was assessed after an APAP overdose of 300 mg/kg administration. Our data showed that the serum levels of ALT, AST and LDH in the APAP group were markedly increased, and were significantly decreased by AG and CGA (40 mg/kg) treatment ($p < 0.05$, Fig.1B, C and D, which suggested that CGA could alleviate liver damage by reducing the activity of ALT, AST and LDH. Moreover, the histopathologic analysis was performed on HE stained hepatic sections. As shown in Fig.1E, APAP administration significantly induced severe hepatocellular injury compared with the control mice, such as loss of hepatocyte architecture, vacuolization of hepatocytes, massive necrosis and mononuclear cell infiltration in the portal area. Conversely, treatment with CGA or AG significantly ameliorated the hepatocellular injury around in the portal area. Compared with the control group, necrosis scores and inflammation scores were increased significantly after APAP administration ($p < 0.05$), while treatment of AG and CGA (40 mg/kg) significantly decreased all the scores ($p < 0.05$, Fig.1F and G), these results suggested that pretreatment with CGA provided a protective role on APAP-induced acute hepatotoxicity in mice.

3.2 Chlorogenic acid suppressed APAP-induced apoptosis of hepatocyte in mice

To further elucidate the role of CGA on APAP-induced apoptosis of hepatocyte in mice, TUNEL staining and western blotting were used. As shown in Fig.2A and B, there were little apoptotic cells in the normal liver tissue. However, significantly increased apoptotic cells could be observed in the APAP group, which was significantly reduced by treatment of AG and CGA (40 mg/kg, $p < 0.05$). To further demonstrate the effect of CGA on APAP-induced apoptosis, the expressions of apoptosis-associated proteins (Bax and Bcl-2) were measured by western blotting. We found that APAP mice presented a significantly elevated

expression of Bax and decreased expression of Bcl-2 ($p < 0.05$, Fig.2C, D and E) and reduced Bcl-2/Bax ratio ($p < 0.05$, Fig.2F) compared to the control mice, which was significantly reversed after treatment with 40 mg/kg of CGA ($p < 0.05$, Fig.2C-F). Taken together, these data indicated that CGA attenuated APAP-induced apoptosis of hepatocyte through regulating Bcl-2 family proteins levels in mice.

3.3 Chlorogenic acid increased autophagy in APAP treated mice

Previous studies have demonstrated that autophagy and apoptosis exist in overlap with many pathophysiology conditions [34]. Enhancing autophagy can prevent the progression of acute liver injury [35]. To verify the effects of CGA on autophagy, immunofluorescence co-localization analysis was firstly used to examine the colocalization of Tom20 (a specific mitochondria marker) and LC3 (autophagy marker). Our data showed that pretreatment of CGA significantly promoted the colocalization of Tom20 and LC3 to exert enhancing autophagy effect compared with APAP mice (Fig.3A). The expression decrease of Tom20 is often considered to be the reduction of mitochondria damage and the activation of autophagy. As shown in Fig.3B, we found that APAP mice presented a significantly elevated expression of Tom20 compared to the control mice, which was significantly reversed after treatment with CGA ($p < 0.05$). Moreover, we tested the expression of autophagy-related genes and proteins (LC3 and p62). As expected, after pretreatment with CGA, the expression of LC3 mRNA and protein were dramatically increased while p62 was the exact opposite compared with APAP mice. Simultaneously, the ratio levels of LC3II/I were significantly decreased in APAP mice while they were remarkably increased in CGA mice ($p < 0.05$, Fig.3C-E). These results indicated that CGA promoted autophagy flux in APAP-induced liver injury mice.

3.4 Chlorogenic acid promoted mitophagy via PINK1/Parkin pathway

As a type of mitochondria-specialized autophagy, PINK1-dependent mitophagy was recently recognized as a novel target for treating alcoholic liver disease and acetaminophen hepatotoxicity, due to its maintaining role in mitochondrial homeostasis [21]. Therefore, we further determined whether CGA promoted PINK1-dependent mitophagy. qRT-PCR and Western blot assay were used to measure the genes and proteins expression levels of the mitophagy marker PINK1 and Parkin, and an obvious inhibition of PINK1 and Parkin expressions were found in APAP mice ($p < 0.05$ or $p < 0.01$, Fig.4A, B and C). Interestingly, pretreatment with CGA at the dose of 40 mg/kg significantly increased the levels of PINK1 and Parkin ($p < 0.05$, Fig.4A, B and C), suggesting that CGA alleviated APAP-induced hepatotoxicity via activating PINK1/Parkin-dependent mitophagy.

4. Discussion

APAP is a commonly used antipyretic and analgesic, which induces acute liver injury at excessive doses, APAP (0.15-3.0 g/kg, po) can cause dose-dependent death in mice [36]. In this study, mice were given 300mg/kg APAP treatment to induce liver injury. Our present study demonstrates that pretreatment with CGA (X) promotes PINK1-dependent mitophagy, (X) inhibits hepatocyte apoptosis through regulating Bcl-2 family proteins under APAP overdose conditions, and (X) reduces mortality in mice and alleviates APAP-

induced acute hepatotoxicity. Collectively, our results were the first to reveal that CGA preserved hepatocyte survival in response to APAP overdose via activating PINK/Parkin mitophagy inhibited hepatocyte apoptosis, indicating CGA might be a promising therapeutic natural polyphenol for the detoxification of APAP-induced liver injury. Graphical summary of the results is schematized in Fig. 5.

It has been well-documented that hepatocyte apoptosis coexisted with necrosis was critically important to acetaminophen overdose induced hepatotoxicity, due to perpetuating liver damage [15, 37]. Conventionally, apoptosis is initiated by mitochondrial dysfunction and regulated by Bax (a proapoptotic protein) and Bcl-2 (an anti-apoptotic protein) [38]. In present study, results from the TUNEL and western blotting analysis showed that CGA significantly decreased the amount of apoptotic cells and reversed the elevated Bax level and decreased Bcl-2 level, clearly indicating that CGA exhibited anti-apoptosis effects on APAP hepatotoxicity.

Natural product protection and prevention of liver injury are particularly well-documented, such as CGA. It has been shown to protect against liver damage caused by different drugs, including carbon tetrachloride, methotrexate, and APAP [25, 26, 31]. Existing studies have shown that CGA has anti-inflammatory and anti-oxidative stress effects in liver injury. Mitochondrial dysfunction, inflammation and oxidative stress are interdependent processes. Mitophagy helps regulate mitochondrial homeostasis by removing dysfunctional mitochondria. Inhibiting mitochondrial autophagy can lead to the accumulation of damaged mitochondria and sometimes the activation of inflammasomes.

Recent evidence suggests that hepatocyte apoptosis overlaps with autophagy in liver injury [39]. Mitochondrial dysfunction leads to insufficient ATP production, increases mitochondrial ROS, excess cytochrome c (Cyt-c) release, upregulation of Bax, caspase-3 and caspase-9, and further initiates the apoptosis pathway [40]. Moreover, hepatocyte apoptosis is regulated by autophagic activity. As expected, we found APAP overdose dramatically inhibited shifts of LC3 protein from cytoplasmic form (LC3^{cyt}) to autophagosome form (LC3^{lys}), and markedly increased p62 and Tom20 level, while CGA treatment reversed the detrimental effects induced by APAP overdose, and the expression of LC3^{lys} on mitochondria increased significantly. Interestingly, the current findings showed that mitophagy played a critical role in removing damaged mitochondria in APAP hepatotoxicity [19], especially parkin-mediated mitophagy [24]. Therefore, it can be speculated that targeting mitophagy-mediated apoptosis is beneficial of relieving acetaminophen hepatotoxicity. In the present study, an obvious inhibition by PINK1 and Parkin expressions were found in APAP mice, which is consistent with previous reports that suppression of Pink1/Parkin augments acetaminophen hepatotoxicity via impairment of hepatic mitophagy [22, 23].

Conversely, CGA significantly activated mitophagy, which was manifested by the increase of PINK1 and Parkin levels. Given all this, these findings provide evidences for the hepatoprotection effects of CGA in activating Pink1/Parkin-mediated mitophagy and inhibiting hepatic apoptosis. In future follow-up studies, we will focus on *in vitro* studies, by administering autophagy inhibitors, and use siRNA to interfere with PINK1 and Parkin, in order to deepen the research on the mechanism of CGA to prevent drug-induced liver injury.

Abbreviations

NAPQI, N-acetyl-p-benzoquinone imine; ROS, reactive oxygen species; GSH, glutathione; APAP, acetaminophen; CGA, Chlorogenic acid; AG, Ammonium glycyrrhizinate; HE, hematoxylin and eosin; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; Cyt-c, cytochrome c.

Declarations

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Author contributions Bang-yan Hu and Jin Li performed the research; Bang-yan Hu wrote the original manuscript; Dao-yin Gong completed histopathology researches; Yan Dai analyzed the data; Shi-jun Xu conceived the study and critically reviewed and revised the paper; Li-hong Wan designed the study and critically edited and revised the paper.

Conflict of interest All authors have no conflict of interest to disclose.

Ethical approval Animal care and experiments protocols were in accordance and approved by the Ethics Committee for Animal Experiments of the Institute of Material Medica Integration and Transformation for Brain Disorders in Chengdu University of Traditional Chinese Medicine and accorded with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Permit No: IBD2019008, Chengdu, China).

Consent to participate Not applicable.

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Figures

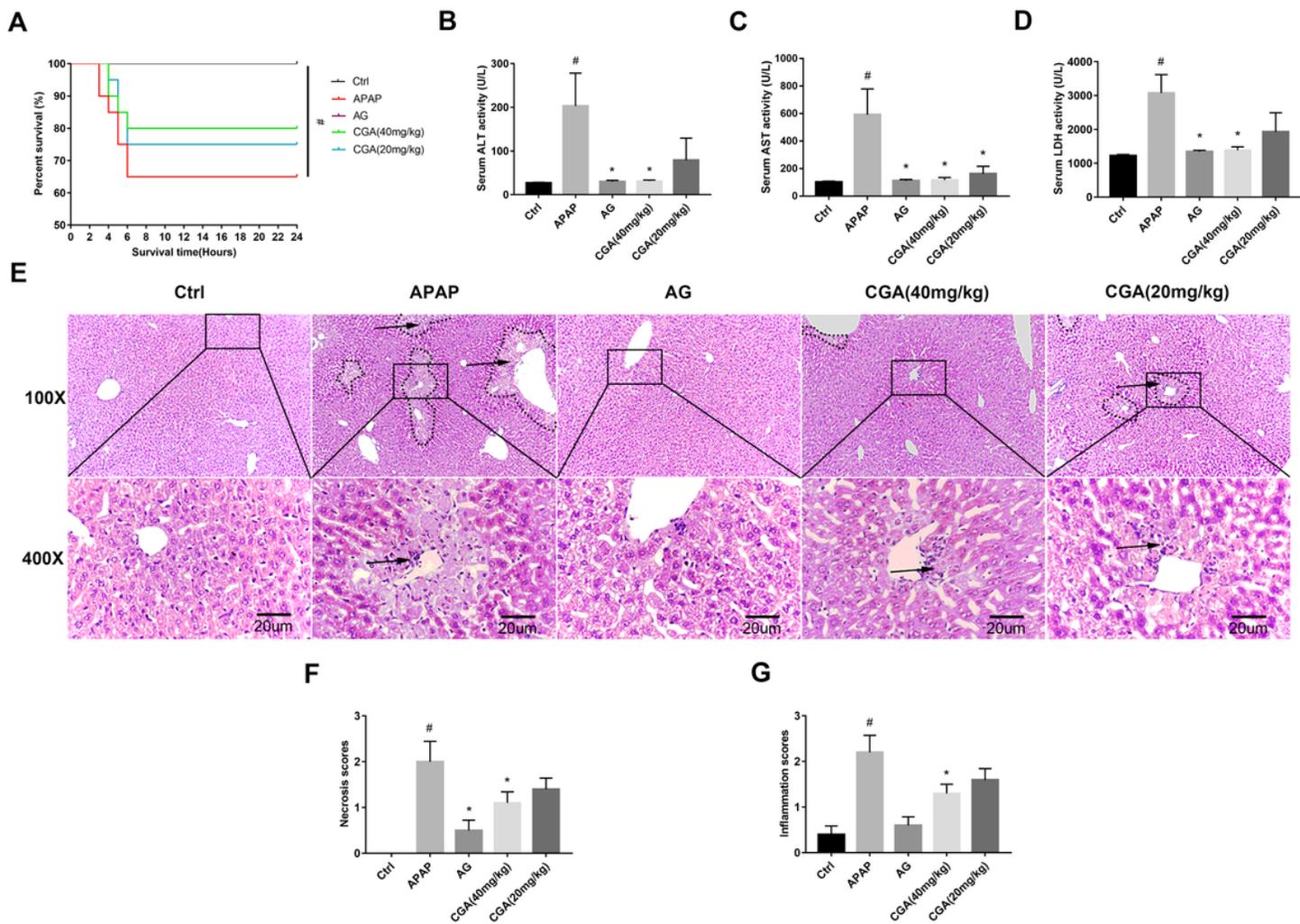


Figure 1

Chlorogenic acid alleviates APAP-induced acute hepatotoxicity in mice (A) The effect of CGA on APAP-induced Survival rate was monitored every hour for 24 hours (Log-rank test, n = 20). (B-D) Serum levels of ALT, AST and LDH (n=5). (E) Representative photomicrographs of liver tissue (H&E staining, 100× and 400×; Scale Bar=20µm); the arrows indicate leukocyte infiltration and the dotted line represent necrotic area, n=5). (F) Histological necrosis scores and (G) inflammation scores were evaluated by an experienced pathologist in a blinded manner under a light microscopy (n = 5). Values are presented as mean ± S.E.M. #p< 0.05 versus Ctrl group; *p< 0.05 versus APAP group.

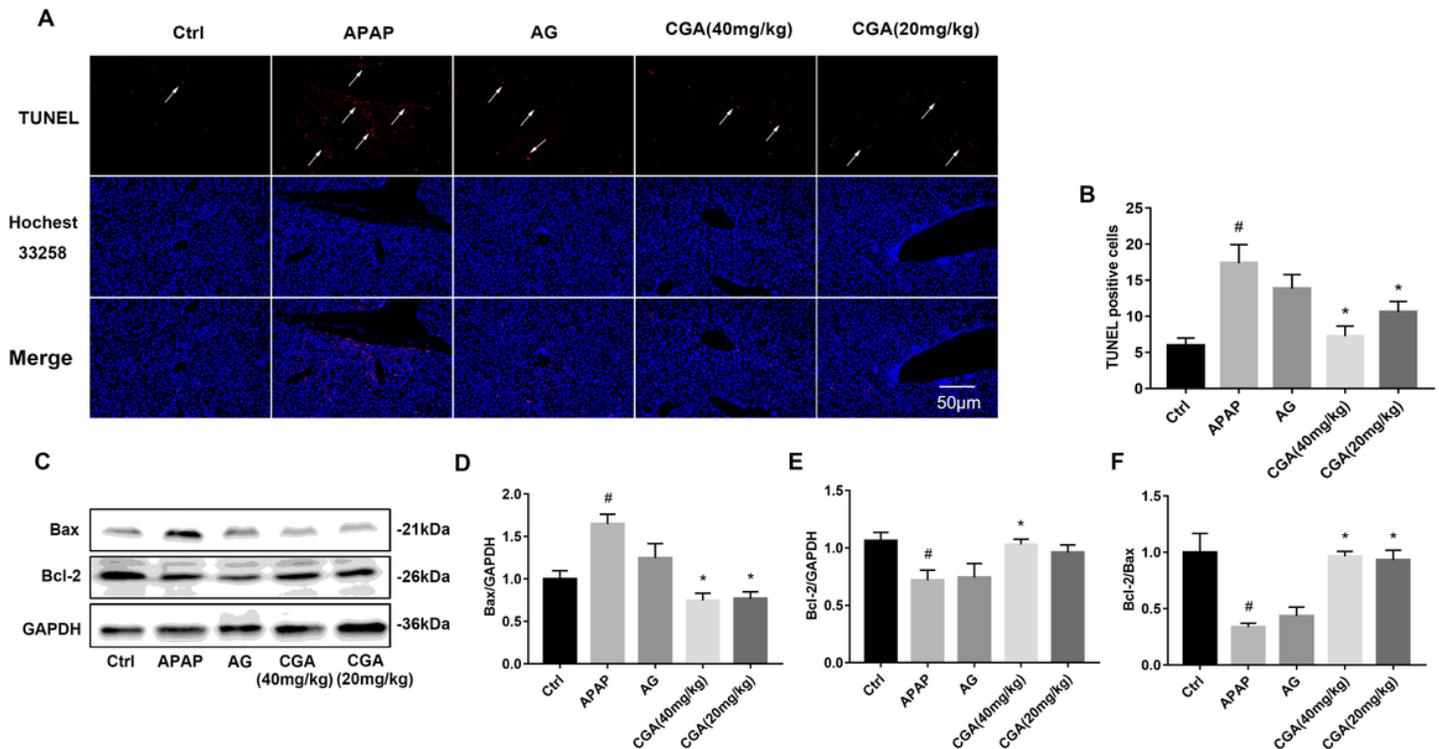


Figure 2

Effects of Chlorogenic acid on acetaminophen-induced apoptosis of hepatocyte (A) Representative images of liver apoptosis (TUNEL assay, 200 \times , Scale Bar=50 μ m); the arrows indicate apoptotic cell. (B) TUNEL-positive cells were manual counted in three randomly selected fields from each slide. (n=4) (C) Representative Western blotting images of Bcl-2 and Bax in liver. GAPDH was used as an internal standard. (D-F) Quantitative analysis of Bcl-2, Bax and ratio of Bcl-2/Bax in liver from different group by Bio-Rad Quantity One v4.62 software. Values are presented as mean \pm S.E.M from three independent determinations. [#]p< 0.05 versus Ctrl group; ^{*}p< 0.05 versus APAP group.

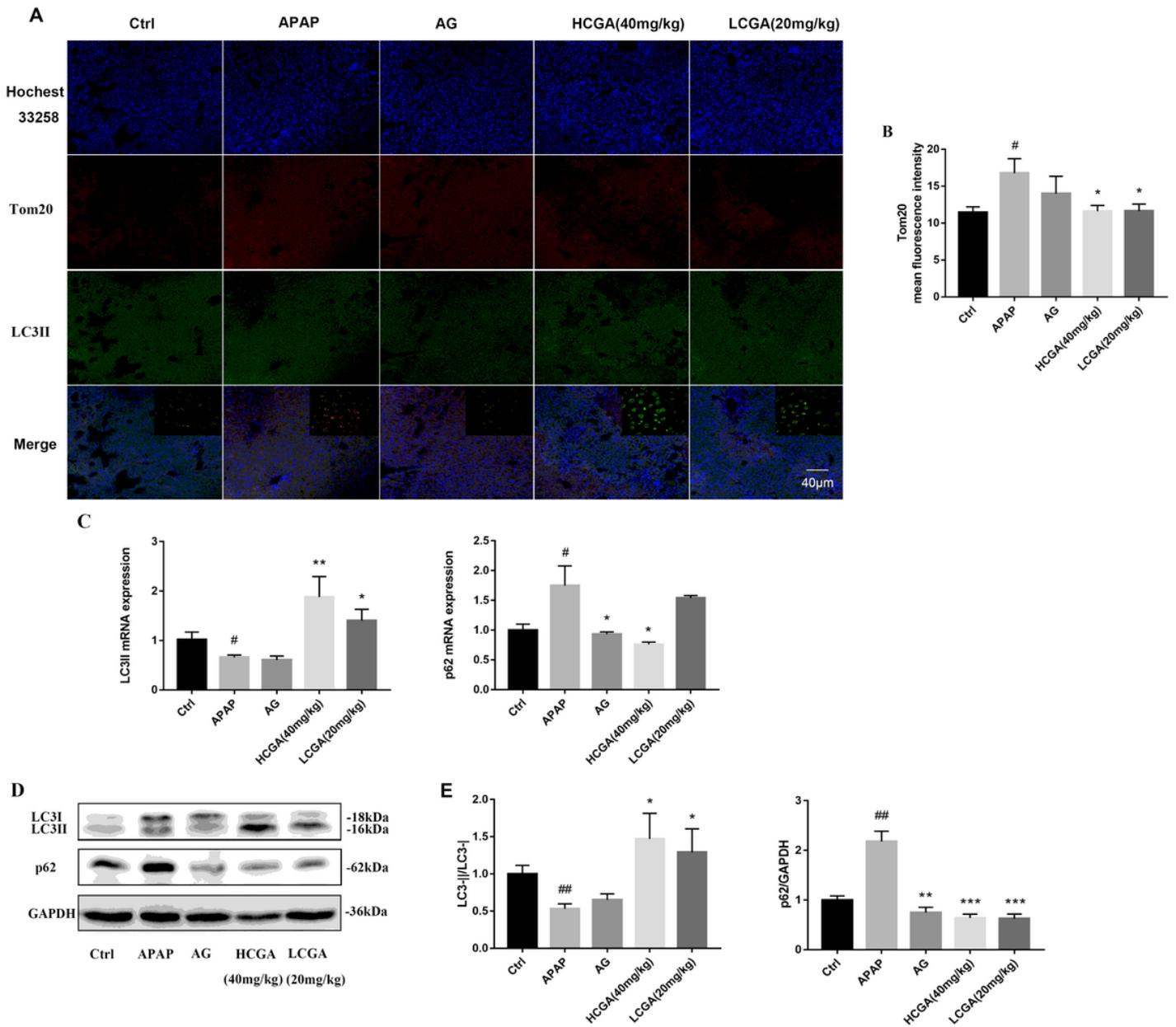


Figure 3

CGA upregulated mitophagy in APAP treated mice (A) Immunofluorescence images of LC3 and Tom20. (n=4, Scale Bar=40 μ m). (B) Quantitative analysis of Tom20 mean fluorescence intensity by Image J. (C) Statistics of LC3 and p62 mRNA expression levels. (D) Representative Western blotting images of LC3 and p62 in liver. GAPDH was used as an internal standard. (E) Quantitative analysis of LC3/LC3 and p62 in liver from different group by Bio-Rad Quantity One. Values are presented as mean \pm S.E.M from three independent determinations. [#]p< 0.05 versus Ctrl group; ^{*}p< 0.05 versus APAP group.

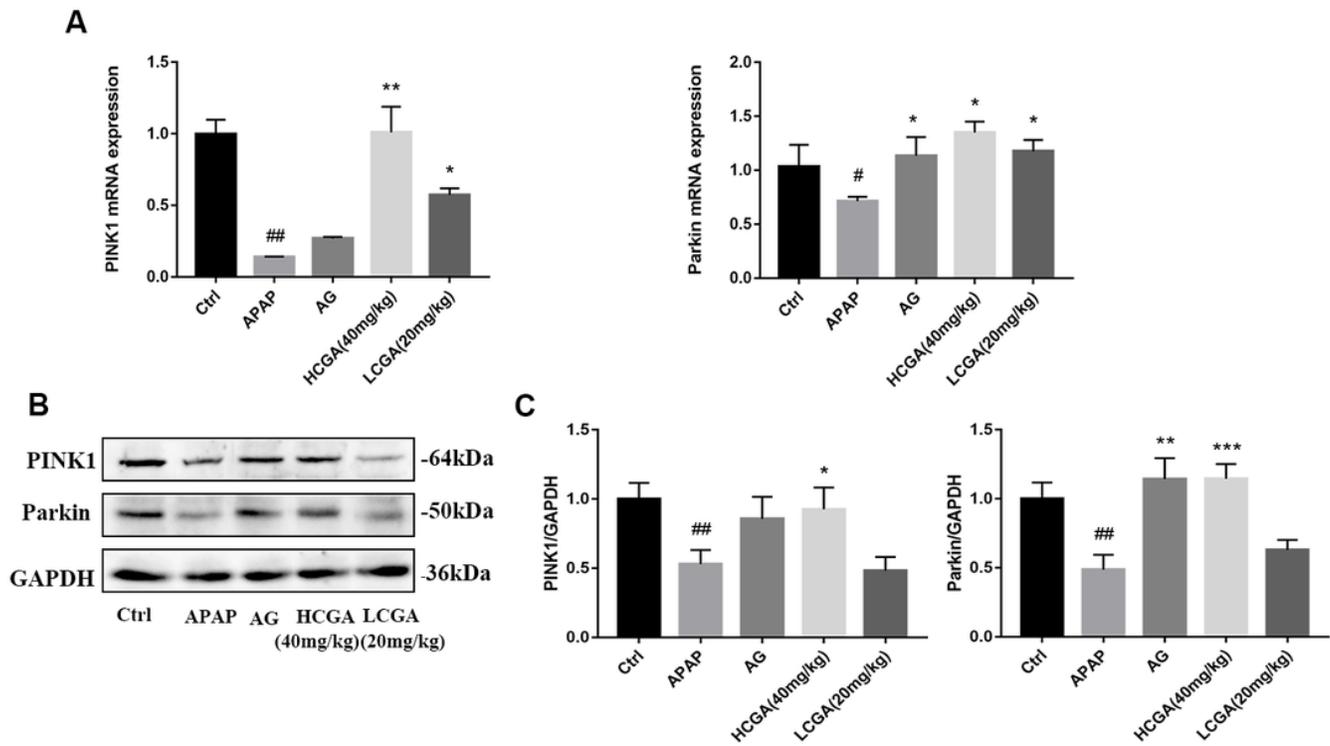


Figure 4

CGA impressed mitophagy via the PINK1/Parkin pathway (A) Statistics of PINK1 and Parkin mRNA expression levels. (B) Representative Western blotting images of PINK1 and Parkin in liver. GAPDH was used as an internal standard. (C) Quantitative analysis of PINK1 and Parkin in liver from different group by Bio-Rad Quantity One. Values are presented as mean \pm S.E.M from three independent determinations. # $p < 0.05$, versus Ctrl group; * $P < 0.05$ versus APAP group.

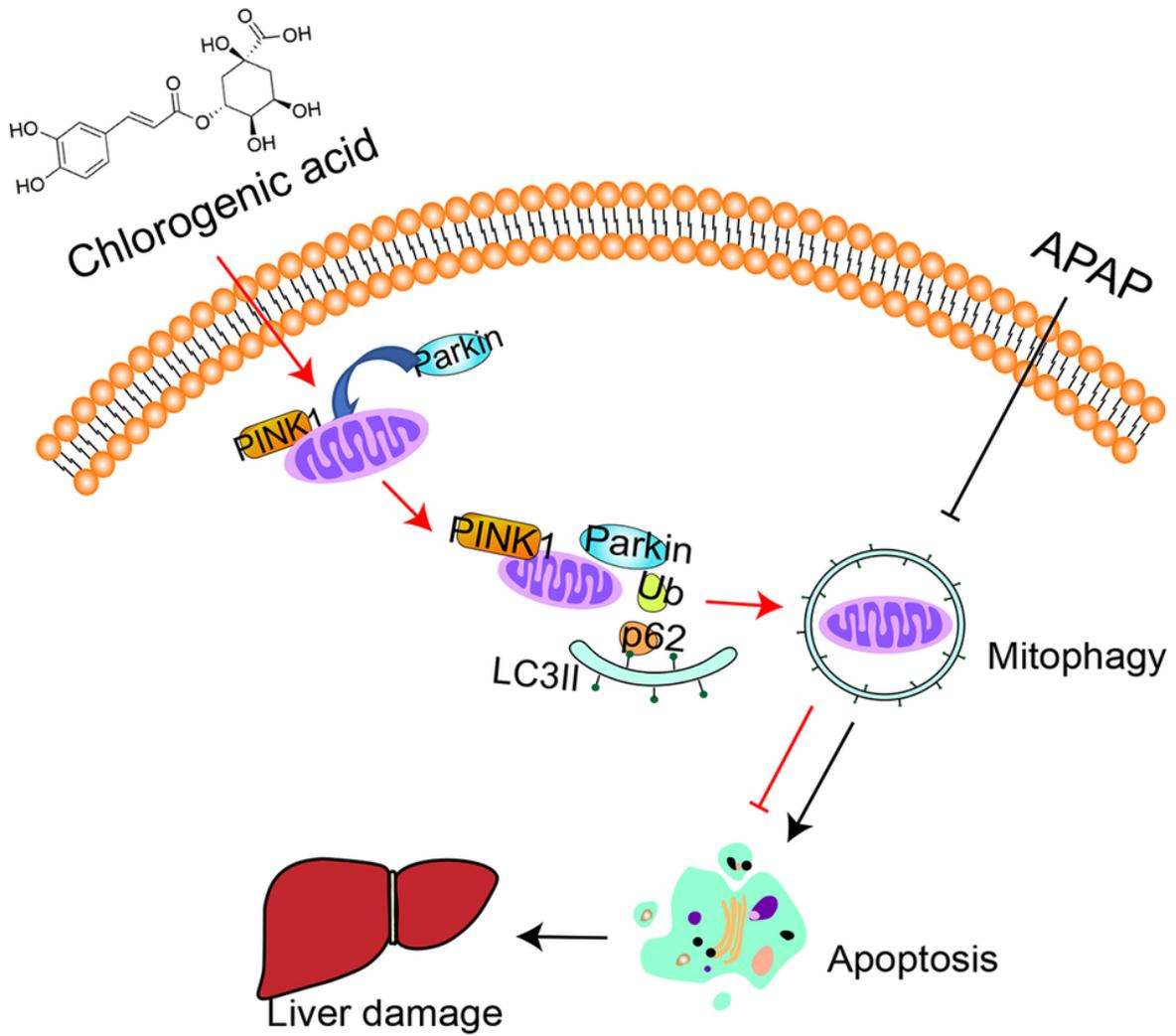


Figure 5

Graphical summary of the results CGA alleviated APAP-induced hepatotoxicity via activating PINK1/Parkin-dependent mitophagy.